Characterisation of the *lex2B* gene and its role in LPS biosynthesis in *Campylobacter jejuni*

A Thesis

submitted in partial fulfillment

of the requirements for the Degree

of

Master of Science in Microbiology

in the

Depertment of Plant and Microbial Sciences

University of Canterbury

By

Dragana Jankovic

University of Canterbury
1999

ACKNOWLEDGEMENTS

I would like to thank the following people for the time, help and support they have given me over the last eighteen months.

Firstly and primarily I would like to thank my supervisor John Klena for his encouragement, help and ongoing support, which were most appreciated. Also many thanks to Jack Heinemann for his input and helpful suggestions.

I am most indebted to Megan Mahan, Vera Andjic and Mattew Walters for their technical expertise, advice and friendship.

Thanks to everyone in John's laboratory especially to David Bean and Scott Godfrey, for their help and friendship over the last year and a half and to members of other laboratories for their additional advice and support.

I would like to thank Marion Savill and Paola Scholes from ESR for their support and never ending encouragement.

A special thanks goes to my mother Milica and sisters Tanja and Vesna for all their support and understanding, without which I would never have made it this far. I would also like to express thanks to Antonija and Dejan Mitrovic for their friendship and moral support.

I would also like to acknowledge and thanks those that helped in the production of this thesis. This includes my friends Biljana Randelovic and Andreja Milenkovic for their unparalleled computer knowledge and expertise.

And last but certainly not the least my partner Milan Gagic for all his support, understanding and help to realise this thesis.

CONTENTS

CHAPTER II: MATERIAL AND METHODS

APPENDIX III:

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVATIONS

APS	ammonium persulphate
bp	base pairs
cfu	colony forming units
ddH_2O	double distilled water
GalNAc	*N*-acetylgalactosamine
GlcNAc	N- acetylglucosamine
HEP	heptose
kb	kilobase pairs
kDa	kilodaltons
kPa	kilopascals
LOS	lipooligosaccaride
LPS	lipopolysaccharide
min	minute(s)
dNTP	2'-deoxy nucleotide triphosphates
OD_{600}	optical density at 600nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
xg	times gravity
SDS	sodium dodecyl sulphate
sec	second(s)
TEMED	N, N, N',N'-tetramethylethylenediamine
UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume
ADP	2'-deoxy adenosine diphosphate

ABSTRACT

Lipopolysaccharides (LPS) are major structural and virulence components of Gram-negative bacteria. Antigenic and phase variation of LPS, the result of changes in carbohydrate composition and assembly of sugars in LPS, leads to increased cell surface diversity. A gene was isolated from *Campylobacter jejuni* and *Campylobacter coli* that had sequence identity to a phase variable gene, *lex2B*, from *Haemophilus influenzae*. The *lex2B* shows sequence similarity to glycosyl and galactosyl transferases, however its function is still unknown.

The aim of study was to characterise *lex2B* in *C. jejuni* and its potential role in pathogenesis of this enteric pathogen. Previous sequence analysis of *C. coli* strain M275 revealed the presence of an ORF whose nucleotide sequence showed a high degree of similarity with *lex2B* from *H. influenzae* and *H. pylori*. The *lex2B* gene from M275 was shown to be transcribed within a LPS biosynthetic locus, downstream of *gmhA* and in an opposite orientation to the *waaF* gene. Nucleotide sequence analysis of an 1.7 kb fragment from *C. jejuni* strain 928 contained in plasmid pDJ4216 revealed a common arrangement of genes, including *lex2B*, between *C. coli* and *C. jejuni*. The *C. jejuni lex2B* ORF was shown to consist of 765 nucleotides encoding a polypeptide of 255 amino acids. Comparison of the *C. coli and C. jejuni lex2B* translated nucleotide sequence revealed strong variation in the second half of the protein. PCR and Southern analysis indicated that the *lex2B* gene in *C. jejuni* appears transcriptionally coupled to adjacent genes and physically linked on the genome to genes involved in biosynthesis of the LPS core. Expression studies based on production of *lex2B* mRNA (RT-PCR) and expression of the putative *Lex2B* protein under transcriptional control of the T7 RNA polymerase in *E.coli* has shown that *lex2B* is capable of synthesing a polypeptide of the predicted size from sequence analysis. In order to create a null mutation in *C. jejuni* 928 by homologous recombination, *lex2B* was cloned into pBluescript II containing a gene encoding resistance to kanamycin in *Campylobacter*. Based on evidence presented in this study, it is speculated that *lex2B* contributes to LPS diversity within *C. jejuni* and *C. coli*.

CHAPTER I

INTRODUCTION

1.1 DESCRIPTION OF THE GENUS

Campylobacter was initially classified as a member of the genus *Vibrio* because of its morphology. In 1963 on the basis of different nucleotide base composition (mol% G+C) and its inability to utilise sugars either oxidatively or fermentatively, a new genus, *Campylobacter,* was created. The genus *Campylobacter* is classified in the family *Spirillaceae* (Veron and Chatelain, 1973).

1.1.1 BASIC MORPHOLOGY AND PHYSIOLOGY

Members of the genus *Campylobacter* are spiral or helical rod shaped bacteria, 0.2 to 0.9 μm wide, 0.5 to 5 μm long, Gram-negative and non-spore-forming (Nachamkin, 1992). Members of the genus express polar, unsheathed flagella and are actively motile using a corkscrew-like motion (Cover and Blaser, 1989). Most *Campylobacter* spp. require a microaerobic atmosphere of 5-7% oxygen, 10% carbon dioxide and 80% nitrogen (Corry *et al.*, 1995; Nachamkin, 1992). While members of this genus replicate across a wide temperature range, the human pathogens have an optimal growth temperature of 42°C. The so-called "thermophilic" *Campylobacter* spp. are *C. jejuni*, *C. coli*, *C. upsaliensis* and *C. lari* (Ketley, 1995).

Under certain stressful environmental conditions, such as exposure to atmospheric oxygen or nutrient limitation, *Campylobacter* spp. undergo a transition in morphology from rods to round or coccoid forms (Ketley, 1995). This transition is associated with the onset of non-culturability on conventional laboratory media (viable non-culturable state (VNC)).

Members of this genus are important animal and human pathogens. However *C. jejuni* is believed to be responsible for 90% of all reported cases of human campylobacteriosis (Wallis, 1994; Stonnet and Guesdon, 1993).

1.1.2 EPIDEMIOLOGY

There are several natural animal reservoirs for *Campylobacter* spp. The organism is a member of the endogenous intestinal flora of a wide range of birds and animals (Altekruse *et al.*, 1994; Blaser and Reller, 1981; Faoagali, 1984; Rosef *et al.*, 1983). These include cattle, sheep, pigs, dogs, cats and poultry. *C. jejuni* has also been isolated from birds such as gulls, migratory ducks, domestic pigeons, crows and Canada geese (Kapperud and Rosef, 1983; Pacha *et al.*, 1988). The major means of transmission however, appears to be via faecal contamination. Potential sources for sporadic cases are hard to determine because of the fastidious nature of the organism and the delay in culture to tracking the source.

Campylobacter spp. sources of infection have been extensively studied in New Zealand and internationally. Four sources account for nearly all epidemic cases: consumption of chicken and chicken products, unpasteurized milk, contact with pets and other animals, and impure water (Eberhart-Phillips *et al.*, 1995). Other food sources implicated in transmission include mushrooms, watermelon, papaya, fish and shellfish (Castillo and Escartin, 1994; Wilson and Moore, 1996; Lacey, 1993).

1.1.3 GENETICS OF THE GENUS *Campylobacter*

Campylobacter spp. have a small genome of about 1.6-1.8 Mb, an exception being *C. upsaliensis*, with a genome of approximately 2.0 Mb (Bourke *et al.*, 1995). *C. jejuni* and *C. coli* chromosomal DNAs are only 36% of the size of the *Escherichia coli* chromosome (4.6 Mb) (Smith *et al.*, 1987). The chromosome of *C. jejuni* is circular and approximately 1.6 Mb (Karlyshev *et al.*, 1998; Sanger centre, 1998). The chromosomal DNA of *Campylobacter* spp. is typified as being extremely A+T rich, with an average G+C content of 30%. In comparasion, the *E. coli* G+C content is 50% (Nuijeten *et al.*,

1990; Chang and Taylor, 1990; Taylor, 1992; Kim *et al.*, 1992; Kim *et al.*, 1993). The small genome size of *Campylobacter* spp. is consistent with their small and fastidious nature; these organisms require numerous supplements for growth. Conjugative plasmids and bacteriophages have been reported in *Campylobacter* and these extrachromosomal elements may contain essential genes required for metabolism (Taylor, 1992).

1.2 *Campylobacter* AS A HUMAN PATHOGEN.

Campylobacter spp. were first recognised in the early decades of this century as a cause of infectious abortion and infertility in sheep and cattle. The first reported case of pathogenicity in humans was in 1947 (Allos and Blaser, 1995). In 1972 after the development of selective stool-culture techniques, *C. jejuni* was recognised as an important cause of acute diarrhoeal disease in humans (Allos and Blaser, 1995). In 1980 campylobacteriosis was made a notifiable disease in New Zealand and since then the incidence of infection has increased yearly (Faoagali, 1984).

1.2.1 PATHOLOGY AND CLINICAL SYMPTOMS OF CAMPYLOBACTERIOSIS

Once established, a campylobacter infection can manifest in several different ways. The vast majority of *C. jejuni* infections are gastrointestinal and usually self-limited, but extraintestinal infections including meningitis, urinary tract infections, reactive arthritis, Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome (MFS) have been reported (Walker *et al.*, 1986; Mishu and Blaser, 1994; Nachamkin *et al.*, 1998).

Gastroenteritis caused by *Campylobacter* spp. can last up to three weeks with an incubation period of 2-11 days. The clinical symptoms include diarrhoea (often bloody), nausea, fever, occasional vomiting, headache, malaise, muscular pain and acute abdominal pain (Stehr-Green *et al.*, 1991; Eberhart-Phillips *et al.*, 1995). Patients generally recover in seven days without treatment, but in up to 20% of cases a relapse or a prolonged illness occurs (Blaser *et al.*, 1983; Reina *et al.*, 1994). Generally hundreds

to thousands of bacteria are required for infection by *C. jejuni*. However, studies indicate that even low doses of *C. jejuni* can produce infection and illness in humans. In one milk-borne outbreak it was estimated that consumption of only 500 organisms resulted in infection (Jones and Telford, 1991b). In a separate study the minimum number of cells ingested by volunteers was 800 organisms, and this resulted in diarrheal illness in >50% of the samples (Black *et al.*, 1988). Conditions which reduce acidity in the stomach, such as surgery or peptic ulcer treatment, increase a person's susceptibility to *Campylobacter* infection. Milk buffers gastric acid and thus fewer organisms are presumably needed for infection (Jones and Telford, 1991b).

Erythromycin remains the treatment of choice for most cases of campylobacteriosis. This agent is characterised by low toxicity, a relatively narrow spectrum of activity and ease of administration (Blaser and Reller, 1981; Allos and Blaser, 1995). Ciprofloxacin has been used as an alternative treatment for acute diarrhoeal cases but bacterial resistance to this agent has increased (Lacey, 1993). *C. jejuni* strains are almost universally resistant to trimethoprim, cephalosporins, penicillin, vancomycin and rifampin (Allos and Blaser, 1995).

1.2.2 EXTRAINTESTINAL *Campylobacter* DISEASE

Extraintestinal diseases can occur as a result of *C. jejuni* infection. The incidence of *C. jejuni* penetrating the intestinal wall and invading a patient's bloodstream resulting in bacteremia is speculated to occur in 0.15% of all intestinal infections (Nachamkin, 1992; Lacey, 1993). *Campylobacter* post-infection complications are infrequent but do occur and are often severe. They include neurological disease such as stroke, meningitis, empyema, encephalopathy and two polyneuropathic conditions, GBS and MFS (Yuki *et al.*, 1994).

GBS has become the most common cause of acute generalised paralysis worldwide with annual incidence internationally of up to 2 cases per 100 000 population (Ropper, 1992; Mishu and Blaser, 1994). Up to two-thirds of GBS cases are preceded by symptoms of an infectious illness, most frequently of the respiratory or gastrointestinal tract (Rees *et al.*, 1993; Rees *et al.*, 1995). Campylobacteriosis is the most common antecedent event

associated with GBS. GBS usually occurs 1-3 weeks after bacterial or viral infections (Ropper, 1992). The mortality rate for GBS is 1-18% while 20% of cases suffer prolonged residual disability (Rees *et al.*, 1993).

GBS is an inflammatory, demyelinating disease of peripheral nerves. Speculation suggests that *C. jejuni*-induced GBS may arise due to the oligosaccharide portion of *C. jejuni* lipopolysacccharide (LPS). It has been observed that LPS from some serotypes of *C. jejuni* mimic the structure of human gangliosides. T and B cells stimulated in response to LPS post-*C. jejuni* infection secrete interleukins and stimulate macrophages (Nachamkin *et al.*, 1998). Macrophages and interleukins are speculated to surround the endoneural vessels and cause demyelination of these neural structures (Lacey, 1993; Salloway *et al.*, 1996). Ganglioside-like structures have been identified at the terminal regions of LPS core oligosaccharides (OS) (Aspinall *et al.*, 1994b). OS structures which have human ganglioside G_{M1} and G_{D1a} structures have been found in serostrains HS:4, HS:19 and HS:41 (Aspinall *et al.*, 1992; Aspinall *et al.*, 1994b; Yuki *et al.*, 1994; Prendergast *et al.*, 1998) and G_{M2}-like OS structures occur in LPS from serotypes HS:1, HS:23 and HS:36 (Aspinall *et al.*, 1992). A *C. jejuni* strain of serotype HS:10, isolated from a patient suffering from MFS, had an OS structure reflecting the terminal region of human gangliosides G_{D3} (Salloway *et al.*, 1996). Chemical analysis of LPS revealed that the core oligosaccharide had a terminal trisaccharide epitope consisting of two molecules of sialic acid (neuraminic acid) linked to galactose. This trisaccharide is not found in cores of non-neuropathic *C. jejuni*. A possible role of this trisaccharide in the etiology of neuropathies has been proposed (Salloway *et al.*, 1996).

1.3 VIRULENCE AND VIRULENCE FACTORS

A reasonable understanding of the general clinical, microbiological and epidemiological aspects of *C. jejuni* infection has been achieved. However, the mechanisms by which *C. jejuni* induces disease are not well-understood (Ketley, 1995; Ruiz-Palacios, 1992). The bacterial virulence factors or determinants required to establish an infection are likely to be multifactorial in nature. Common mechanisms postulated for bacterial-induced illness include: adherence, production of enterotoxins, replication, colonisation and

resistance against host defences (e.g., lysozyme, phagocytes, complement). Additionaly some microorganisms, such as *C. jejuni*, must invade and proliferate within the intestinal epithelium. This results in cellular damage and activitation of the host inflammatory response (Ruiz-Palacios, 1992; Ketley, 1997; Cover and Blaser, 1989).

In this section I will discuss factors produced by *C. jejuni* that are implicated or proven to be important for the pathogenicity of the organism. These factors (flagella, outer membrane proteins (OMPs), LPS, cytotoxins and iron aquisition) are briefly examined below.

1.3.1 CHEMOTAXIS AND MOTILITY

Effective colonisation of host tissues requires chemotaxis. *Campylobacter* possesses cellular mechanisms that detect chemical gradients and have linked motility functions that enable the cells to move up or down the gradient. Hugdahl *et al.* (1988) have demonstrated that several host cell molecules may be chemoattractants, including mucin, L-serine and L-fucose, while several bile acids have chemorepellant effects. However, little is known regarding the molecular basis of *Campylobacter* chemotaxis. One regulatory protein, CheY, has been characterised. Alterations in the amount of CheY, a protein that mediates clockwise rotation of flagellum, affects *in vitro* invasion and *in vivo* virulence (Yao *et al.*, 1997).

Colonisation requires flagella, the best characterised virulence determinants of *C. jejuni* (Ketley, 1995). Aflagellated mutants and flagellated nonmotile *C. jejuni* strains cannot colonise animal models (Taylor, 1992; Wallis, 1994; Ketley, 1997). *C. jejuni* flagella are unsheathed and demonstrate phase and antigenic variation. Flagella are also highly immunogenic, with patients producing antibodies to the flagella shortly after infection (Taylor, 1992). Phase variation refers to a bi-directional transition between flagellated and aflagellated phenotypes. Mutation analysis revealed that these modifications of flagellin may play a role in the protective immune response (Guerry *et al.*, 1996; Ketley, 1997). Phase and antigenic variation may alter the ability of a bacterium to invade host cells and to resist killing by antibodies and complement (Moran *et al.*, 1994; van Putten, 1993; Weiser *et al.*, 1989). Reported mechanisms for phase and antigenic change are

slipped strand mispairing (Kimura and Hansen, 1986; Moxon and Maskell, 1992; Cope *et al.*, 1991; Preston *et al.*, 1996; Inzana *et al.*, 1997) and post-translation modification of the flagellin protein by glycosylation (Constantinidou *et al.*, 1996; Doig, *et al.* 1996).

1.3.2 ADHESINS

The ability of many pathogenic bacteria to bind to non-professional phagocytic cells is an important virulence determinant as it prevents the colonising bacteria from being swept away by mechanical cleansing forces such as peristalsis and fluid flow. Bacteria adhere only to complementary surfaces, and adherence involves an interaction between structures on the surface of bacterium (adhesins) and receptors on the substratum. Adhesins are proteins that engage in protein-carbohydrate or protein-protein interactions, targeting structures contained in matrix glycoproteins, integral membrane glycoproteins, or glycolipids (Cundell and Tuomanen, 1995). It remains formally possible that adhesins are carbohydrates that engage cognate carbohydrates, as suggested in a few eucaryotic interactions (Turley and Roth, 1980). Possible adhesins include Omps, flagellae and LPS (McSweegan and Walker, 1986).

Using a ligand binding assay, De Melo and Peche're identified four Omps that may play a role in mediating *C. jejuni* binding to host cells (De Melo and Peche're, 1990). One Omp, of a 28 kDa periplasmic binding protein (PEB1), is involved in amino acid transport (Pei and Blaser, 1993). Several authors have reported PEB1 as the major adherence factor conserved in all *C. jejuni* and *C. coli* isolates, but all studies have so far lacked *in vivo* evidence of this (Burnes *et al.*, 1995; Gavis *et al.*, 1996). Recently Konkel and colleagues identified an adhesin termed CadF (*Campylobacter adhesion to fibronectin*). The CadF protein is conserved among *C. jejuni* and *C. coli* isolates (Konkel *et al.*, 1997; Konkel *et al.*, 1999a). It is speculated that binding of *C. jejuni* to fibronectin is an important early event that leads to successful colonisation. Following binding, *C. jejuni* would have sufficient time to synthesise the proteins required to facilitate internalisation. Production of fimbriae has not been observed in *C. jejuni* (Walker *et al.*, 1986) but production of an environmetally regulated pilus-like appendage has been reported in response to bile rich environments (Doig *et al.*, 1996).

There is some evidence that flagella also function as an adhesin, enabling *C. jejuni* to attach to epithelial cell surfaces (Field *et al.*, 1993; McSweegan and Walker, 1986). Other researchers have refuted this finding (Ketley, 1995; Konkel and Klena, 1995). It has been speculated that *C. jejuni* adhesion to host cells may not be necessary as the bacterium is able to remain highly motile in mucus (McSweegan and Walker, 1986; Ketley, 1995).

McSweegan and Walker suggested that LPS may be an important adhesin. Their results indicate that the short O side chains of *C. jejuni* strain HC were important in mediating contact with INT 407 epithelial cells. LPS was extracted from radiolabelled *C. jejuni* and used in an INT 407 cell adhesion assay. Results showed that LPS specifically bound to cells, suggesting the role of LPS in attachment to epithelial cells. Periodate oxidation of LPS reduced the binding of *Campylobacter* to cells, suggesting that the binding is through the carbohydrate portion of the LPS molecule (McSweegan and Walker, 1986). Purified LPS from the *C. jejuni* strain HC was also effective in blocking the attachment of intact HC cells to INT 407 cells.

1.3.3 AVOIDANCE OF IMMUNE SYSTEM

1.3.3.1 Resistance to complement-mediated killing

The complement system plays a key role in response of the host to microbial invasion and infection. A large number of Gram-negative bacteria are susceptible to complement-mediated killing, and exposure of these cells to a suitable source of complement, such as serum or plasma, may lead to a rapid and efficient reduction in viability (Taylor, 1995). Killing is sometimes accompanied by lysis of the target bacteria by lysozyme. LPS O side chains are implicated as a major structural determinant for complement resistance (Joiner, 1985). Mutations resulting in transition from O^+ to O^- phenotypes lead to large increases in complement susceptibility (Gamian *et al.*, 1992). Gram-negative organisms, which have smaller or nonexisting O antigens, use other carbohydrate structures for complement resistance. For example, phase variation of surface antigens is a common theme for some bacteria to evade the immune system (e.g., *Haemophilus influenzae*

(Moxon and Maskell, 1992; Jarosik and Hansen, 1994), *H. somnus* (Inzana *et al.*, 1997), *Neisseria meningitis* and *Neisseria gonorrhoeae* (van Putten, 1993). One genetic mechanism for LPS phase variation is based on slipped strand mispairing. Gene loci that have highly repetitive DNA sequences are subject to slipped-strand mispairing, causing recombination-independent deletions or insertions of entire repeat units (Levison and Gutman, 1987). It is believed that other mechanisms are also responsible for variation such as LPS sialylation (Mandrell *et al.*, 1993; Rest and Mandrell, 1995). How *Campylobacter* avoids complement-mediated killing is still unknown. *C. jejuni* and *C. coli* are generally serum-sensitive in the absence of specific antibody, but isolates from blood are less sensitive than those from stool (Walker *et al.*, 1986). Complement-opsonized *C. jejuni* are efficiently killed by polymorphonuclear leucocytes, whereas without complement-opsonisation, phagocytosis is less efficient and strain dependant (Pennie *et al.*, 1986; Walan *et al.*, 1992; Autenrieth *et al.*, 1995). Possible factors used to evade complement are variation in structure of LPS (core sugars and O-antigen) and LPS sialyation (van Putten, 1993).

C. jejuni antigen variation and invasion

C. jejuni LPS consists of a low molecular weight fraction and in some cases it may also contain a high molecular weight fraction (Preston and Penner, 1989; Mills *et al.*, 1992; Aspinall *et al.*, 1992). Many *C. jejuni* serovars , e.g., HS:1, HS:2, HS:4, HS:19, HS:23, and H:36, do not display LPS with an extensive O side chain (Aspinall *et al.*, 1994; Yuki *et al.*, 1994). The presence of a rough LPS phenotype in *C. jejuni* may be a significant factor for its ability to colonise the epithelium, unlike strains with a smooth LPS phenotype (Ketley, 1997; McSweegan and Walker, 1986). The basolateral epithelial cell surface of the intestine appears to be the preferred binding site of both the flagellum and LPS, suggesting that the epithelial cell surface contains better *Campylobacter* receptors than does the intestinal mucus (McSweegan and Walker, 1986). *Campylobacter* may cross the intestinal epithelium by translocation or epithelial cell invasion followed by cell lysis (Konkel *et al.*, 1992). An additional epithelial translocation pathway could be via M cells (Walker *et al.*, 1988; Walker *et al.*, 1992).

After colonisation of the mucus, *Campylobacter* cells disrupt the normal absorptive capacity of the intestine by damaging epithelial cells either by cell invasion, the production of toxin(s), or both (Wallis, 1994). The occasional bacteraemia case (ladron de Guevara *et al.*, 1994), and inflammation of the intestine strongly suggest that cell invasion is an important pathogenic mechanism. Recently Konkel *et al.* (1999b) have identified a protein required for *C. jejuni* internalisation by non-professional phagocytic cells. This protein, CiaB, may be secreted into INT 407 cells via a type III secretion system that stimulates host cell signalling and triggers bacterial internalisation (Konkel *et al.*, 1999b). A null mutation in *ciaB* results in a non-invasive phenotype and abolishes the secretion of all contact-dependent secretory proteins.

The role of the flagella in colonisation and invasion has been confirmed with defined genetic mutants (Taylor, 1992; Nachamkin *et al.*, 1993; Wassennaar *et al.*, 1993; Yao *et al.*, 1994). The *flaAflaB*$^-$ mutants demonstrated >55% reduction in adhesion and 97% reduction of invasion into tissue culture cells comparing with wild types. The *ptlA* (paralysed flagella) mutants showed abberation in flagellar structure at the point at which the filament attaches to the cells. These mutants displayed the same, non-invasive phenotype, as *ciaB*$^-$ with the level of adhesion just two fold reduced (Yao *et al.*, 1994; Konkel *et al.*, 1999b). This indicated that *Campylobacter* invade by processes requiring active motility.

1.3.3.2 Toxins and nutrient aquisition

Toxins may also contribute to the disease process. *C. jejuni* isolates have been reported to produce various toxic activities, including a heat labile enterotoxin and several cytotoxins (Walker *et al.*, 1986; Suzuki *et al.*, 1994), including a cholera-like toxin, cytolethal distending toxin, a shiga-like toxin and an haemolysin (Wallis, 1994; Ketley, 1995). However, other studies have questioned the significance of these findings (Konkel *et al.*, 1992).

LPS is an essential component of bacterial cell-associated toxins (endotoxins) (Schnaitman and Klena, 1993; Raetz, 1993). Endotoxins can elicit disease symptoms ranging from chills and fever to irreversible shock and death. Paradoxically, these same

endotoxins can enhance the body's overall immune resistance to bacterial and viral infections and cancer (Rietschel and Brade, 1992). The lipid A moeity possesses the majority of the endotoxic activities associated with LPS.

In order to colonise the intestine, *Campylobacter* must be able to compete with resident flora for nutrients, including iron, and avoid the host immune defences. Iron is an essential element for all living organisms and pathogenic bacteria must obtain iron throughout the infectious process. In host tissues, the availability of iron is very low, as most iron is complexed with host proteins such as hemoglobin and transferrin. *Campylobacter* have not been shown to produce siderophores (specific chelators for ferric ions), but they are able to use exogenous siderophores (Field *et al.*, 1986; Richardson and Park, 1995). Iron homeostasis in *C. jejuni* is under the regulation of the Fur protein as it is in most Gram-negative bacteria (Wooldridge *et al.*, 1994; van Vliet *et al.*, 1998). The growth rate of a *C. jejuni fur* mutant has been shown to be reduced compared to that of the wild-type under both low- and high-iron conditions (van Vliet *et al*, 1998).

1.4 OVERVIEW OF LIPOPOLYSACCHARIDE

The surface of Gram-negative bacteria is an important entity in environmental and host-parasite interactions. The outer membrane protects cells from the detergent action of bile salts, degradation by digestive enzymes present in intestinal tracts and antibiotics (Nikaido and Vaara, 1985). Another important function of the outer membrane is to endow the bacterial surface with strong hydrophilicity. This is an important feature which enables cells to evade phagocytosis, complement, and to avoid specific immune attack (Nikaido and Vaara, 1985). LPS are essential components of the outer membrane of Gram-negative bacteria; they are the immunodominant antigens on cellular surfaces, and are the major constituent of bacterial endotoxin (Stanier *et al.*, 1984).

LPS molecules are conserved in overall structure. LPS is composed of three functional domains, a hydrophobic glycolipid known as lipid A that anchors the LPS to the outer

membrane, a hydrophilic non-repeating core OS consisting of an inner and outer region, and a distal repeating polysaccharide, known as the O polysaccharide, presented at the cell surface (Reeves, 1994a). Lipid A and the core OS are synthesised and translocated together as a single unit which serves as an acceptor for pre-formed O antigen to yield the completed LPS molecule (Whitfield, 1995).

1.5 BIOCHEMISTRY AND ORGANISATION OF LPS BIOSYNTHETIC GENES

Enterobacteriaceae polysaccharide biosynthetic pathways that have been studied at a genetic level are characterised by the organisation of biosynthetic genes into loci consisting of one or more operons (Daniels *et.al.*, 1992; Jiang *et.al.*, 1991; Schnaitman and Klena, 1993). Each loci contains most of the genes that are necessary for the synthesis of the corresponding component of a particular polysaccharide. Several loci such as these have been found (Figure 1). In addition to the *waa* and *wba* loci for LPS biosynthesis, these include the *wec* gene loci, which is comprised of genes encoding enzymes for the biosynthesis of the enterobacterial common antigen (ECA) (Daniels *et.al.*, 1992), and the *cps* and *kps* gene loci, comprised of genes encoding enzymes involved in the synthesis of type I capsules such as colanic acid (Keenleyside *et.al.*, 1993) and type II capsules (Reeves *et.al.*, 1996). In this section I shall briefly discuss the gene loci of *E. coli* K12, *S. typhimurium* LT2 and *C. jejuni* that are involved in LPS biosynthesis, and their organisation.

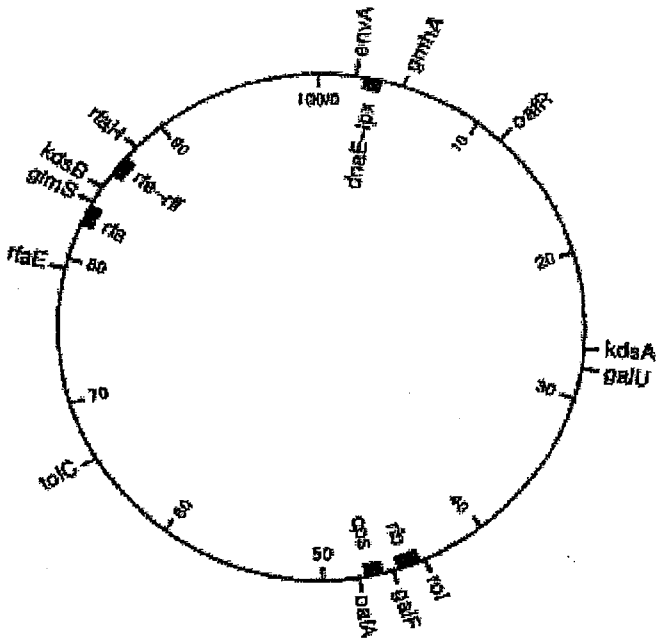


Figure 1. Composite genetic map showing approximate locations of genes and gene loci (solid blocks) involved in LPS biosynthesis that have been identified in *E. coli* and *S. typhimurium*. Numbers indicate position in minutes. This map has been modified to accommodate the different genetic maps of these two organisms (Adapted from Schnaitman and Klena 1993; *gmhA* gene position mapped with information from Brooke 1996)

1.5.1 LIPID A

Lipid A is a non-conventional hydrophobic glycolipid. Lipid A of *E. coli* and *S. enterica* sv. Typhimurium LT2 (hereafter referred to as *S. typhimurium* LT2) is a β,1-6 linked disaccharide of N-acetylglucosamine (GlcNAc) to which five or six fatty acid residues are attached through amide and ester linkages, respectively (Figure 2) (Raetz, 1993). The unique amide-linked fatty acids are 3-hydroxytetradecanoic acids; however, these may be replaced by other 3-hydroxy acids or 2-hydroxy fatty acids in other Gram-negative bacteria such as *Brucella* spp. (Nikaido and Nakea, 1979), *Pseudomonas aeruginosa* (Raetz, 1990), *Neisseria gonorrhoeae* (Goldman *et.al.*, 1987; Takayama *et.al.*, 1986). Fatty acids isolated from *C. jejuni* were 3-hydroxytetradecanoic, hexadecanoic and tetradecanoic acids (Conrad and Galanos, 1990; Moran *et.al.*, 1991a; Aspinall *et.al.*, 1995). Genes encoding several of the enzymes known to be involved in lipid A biosynthesis are located in a small cluster of 11 genes organised into a complex

operon, which has been termed the macromolecular synthesis II operon (Coleman and Raetz, 1988). Three of the genes for lipid A biosynthesis, *lpxA*, *lpxB* and *lpxD* map to 4 minutes with *fabZ* and several other genes involved in macromolecular synthesis (Reeves, 1994b). Sequence analysis of *C. jejuni lpxA* and *fabZ* suggests that the essential order of these genes is the same as in *E. coli* K12 (Ibbitt, 1997). Other *E. coli* genes such as *dnaE* (encoding the α core subunit of DNA polymerase III) also map to this operon. Another gene, *lpxC* (previously known as *envA*) whose product is known to be involved in lipid A biosynthesis is located (2 min) in a gene cluster encoding for enzymes involved in cell division (Reeves, 1994b).

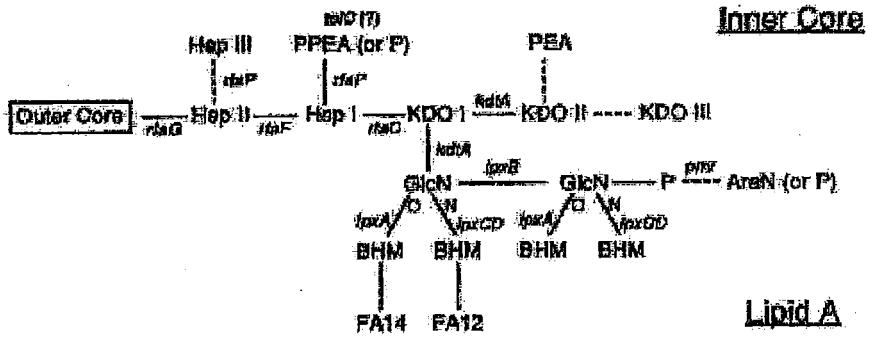


Figure 2. Carbohydrates that compose lipid A and inner core regions of enteric bacteria and genes involved in their synthesis. The lipid A-inner core region is a composite of *E. coli* K12 and *S. typhimurium* structures.

Abbreviations:
BHM =β-hydroxymyristate; FA12=laurate; FA14=myristate; P=phosphate;
PEA= phosphorylethanolamine; PPEA =pyrophosphorylethanolamine; GlcN =glucosamine;
AraN =4-aminoarabinose

The lipid A component of *C. jejuni* LPS has unusual chemical properties in comparison with that of *E. coli* and *Salmonella* spp. Using high voltage paper electrophoresis, serological, structural and chemical techniques, structurally unique lipid A molecules containing monosaccharide or disacccharide backbones composed of other amino sugars such as 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) or glucosaminuronic acid (Weckesser and Mayer, 1988) have been characterised. *C. jejuni* lipid A contains both GlcN and GlcN3N (Moran *et.al.*, 1991b; Moran, 1995). Previous work by Moran and coworkers has shown that *C. jejuni* LPS contains three lipid A backbones composed of GlcN-GlcN, GlcN3N-GlcN3N, and a hybrid disaccharide GlcN3N-GlcN (Moran, 1995).

Changes in the molecular structure of lipid A due to diaminoglucose substitution of the hydrophilic backbone and length of the non-hydroxylated fatty acids residues attached to lipid A may lower the endotoxic potency of the *C. jejuni* LPS molecule.

1.5.2 OLIGOSACCHARIDE CORE

The hydrophilic non-repeating oligosaccharides of most Gram-negative bacteria studied have an inner domain composed of KDO and L-glycero-D-mannoheptose (Hep) to which an outer domain composed of hexoses (e.g., glucose, galactose) and GlcNAc is sequentially added. Phosphate and phosphoethanolamine moieties may also be present in substoichiometric amounts (Reeves, 1994b).

1.5.2.1 Inner domain

The inner core is highly conserved across diverse genera. *Campylobacter* are no exception to this rule. Among all *C. jejuni* serotypes the inner domain is conserved and contains a Hep-1(β-D-Glucose 1-4 linked branch)3-Hep-1-5-KDO linkage (Aspinall *et.al.*, 1993b).

KDO

Attached to the non-reducing end of lipid A is the OS core region. The core is functionally divided into an inner and outer portion. The inner core is composed of at least two residues of 3-deoxy-D-manno-octulosonic acid (KDO) usually followed by two residues ADP-L-glycero-D-mannoheptose (ADP-Hep) (Sirisena *et.al.*, 1992; Schnaitman and Klena, 1993). The eight-carbon acid KDO is linked α(2-6') to the lipid A disaccharide (Figure 2). This attachment is catalysed by the enzyme KDO transferase, which is encoded by the *waaA* gene in *E. coli* (Belunis, 1995). In *E. coli*, strains that are defective in lipid A or KDO biosynthesis have been isolated as conditional lethal mutants, suggesting the minimal structure for survival is lipid A-KDO. An *H. influenzae* mutant, Rd:169, lacking all core sugars except a single phosphorylated KDO, has been characterised. This mutant is able to grow at normal rates *in vitro* despite having the "roughest" LPS described to date (Zamze *et.al.*, 1987).

In contrast to other LPS biosynthetic loci, genes involved in the biosynthesis of KDO (*kdsA*, *kdsB* and *waaA*) are unlinked on the *E. coli* chromosome even though their gene products take part in the same biosynthetic pathway (Schnaitman and Klena, 1993). *kdsA* is transcribed from its own promoter located at approximately 27 min. The *kdsB* gene maps to 84.7 minutes and is thought to be the proximal gene of another small operon (Goldman *et.al.*, 1987). The *waaA* gene encoding the KDO transferase is located at the end of the *waa* cluster at approximately 81 minutes (Belunis, 1992; Schnaitman and Klena, 1993).

KDO is a promising target in the design of novel antibacterial agents as the presence of KDO has not been reported in mammalian cells (Hammond *et.al.*, 1987).

Heptose

The presence of two or more Hep residues in the inner core is highly conserved in Gram-negative bacteria. ADP-Hep molecules are added sequentially to KDO I of the newly synthesised KDO_2-Lipid A. Attachment of Hep I is through an α glycosidic linkage at position 5 of KDO I, while the second Hep is attached α(1-3) to Hep I. A branch Hep, Hep III, if present, is linked α(1-7) to Hep II by a specific LPS heptosyl transferase (Reeves, 1994b). At least seven genes have been identified whose products are involved in the synthesis of the heptose region of the LPS core of *S. typhimurium*. These include four structural genes involved in biosynthesis of Hep (*gmhA*, *gmhB*, *gmhC*, *gmhD*), three heptosyl transferases (*waaC*, *waaF*, *waaQ*) and genes *waaP* and *waaY* whose products are necessary for LPS phosphorylation and crosslinking (Parker *et.al.*, 1992; Heinrichs *et.al.*, 1998a).

Eidels and Osborn (1971) were the first to propose a biosynthetic pathway for ADP-Hep. This pathway is composed of four enzymatic steps and requires sedoheptulose-7-P, a product of the pentose phosphate pathway (Eidels and Osborn, 1971). According to Eidels and Osborn, the conversion of sedoheptulose-7-P to D-glycero-D-mannoheptose-7-P occurs by a phosphoheptose isomerase. Conversion of D-glycero-D-mannoheptose-7-P to D-glycero-D-mannoheptose-1-P by a phosphoheptose mutase is proposed as the second step of the pathway. D-glycero-D-mannoheptose-1-P is charged with ATP

generating ADP-D-glycero-D-mannoheptose and pyrophosphate (PPi) by an ADP-Hep synthase. In the last step racemisation by an epimerase changes ADP-D-glycero-D-mannoheptose to the ADP-L-glycero-D-mannoheptose isomer. The completed Hep molecule is then used as a substrate for addition to inner core by a specific heptosyl transferase (Eidels and Osborn, 1974; Brooke, 1996).

A phosphoheptose isomerase encoded by the *gmhA* catalyses the first step in Hep biosynthesis in *E. coli*, *C. jejuni* and *C. coli* (Brooke, 1996; Upritchard, 1997). The *gmhA* gene maps to 5.3 minutes on the *E. coli* chromosome, distal to the macromolecular II synthesis operon (Brooke, 1996). The translated *gmhA* amino acid sequence shows similarity to a family of conserved aldo/keto isomerases. The GmhA from *C. coli* strain M275 has 62% identity with *E. coli* GmhA and 92.3% identity with *C. jejuni* strain NCTC11168 (Upritchard, 1997). The organisation of *gmhA* on the *C. jejuni* and *C. coli* chromosome appears to be different to that reported for *E. coli* and *H. influenzae*. In *C. coli* M275, *gmhA* is linked downstream to a gene known as *lex2B* (a putative hexosyl transferase) and *waaF* (heptosyl transferase II). This arrangement is novel and has not been reported for any other Gram-negative organism to date (Upritchard, 1997). The presence of *lex2B* in this arrangement is a variable feature for *C. jejuni* and *C. coli* isolates. Sequence analysis revealed the absence of *lex2B* in some isolates of *C. jejuni* (e.g. NCTC11168 strain) (Upritchard, 1997; Sanger centre, 1998). In other bacteria such as *H. influenzae* (Jarosik and Hansen, 1994), *H. somnus* (Inzana *et.al.*, 1997), *N. gonorrhoeae* (Erwin *et.al.*, 1996), *N. meningitis* (Jennings *et.al.*, 1995) and *H. pylori* (Alm *et.al.*, 1999), the *lex2B* homolog is always present on the chromosome and has been shown to play a role in LOS phase variation.

The genes *waaC*, *waaF*, and *waaQ* have been identified as encoding heptosyl transferases I, II, III for transfer of Hep to the inner core (Siresena *et.al.*, 1994; Heinrichs *et.al.*, 1998b). Heptosyl transferase I is encoded by the *waaC* gene and is responsible for addition of the α(1-5) linked Hep I residue to KDO I in *E. coli* (Chen and Coleman, 1993), *S. typhimurium* (Sirisena *et.al.*, 1992) and *C. jejuni* and *C. coli* (Klena, 1998). *In vitro* evidence revealed that mutants in *waaC* contained an ADP-Hep precursor but isolated LPS lacked Hep and was structurally identical to KDO_2-lipid A.

Functional interspecies complementation has demonstrated that *waaC* isolated from *E. coli* and transfer into defective *S. typhimurium* and *H. influenzae waaC* mutants, rebuilds full LPS (Sirisena *et.al.*, 1992; Chen and Coleman, 1993). The *waaC* gene isolated from *C. jejuni* and *C. coli* successfully restores full length LPS in *S. typhimurium waaC* mutants (Klena, 1998). This suggests the proteins are functionally conserved and biosynthesis of *Campylobacter* LPS occurs by a similar mechanism to that of the enteric LPS.

The *waaF* gene has been identified as encoding heptosyl transferase II which adds the second α(1-3) linked heptose to the main nascent chain of the inner core (Schwan *et.al.*, 1995; Sirisena *et.al.*, 1994). Point mutations in the *waaF* gene of *S. typhimurium* LT2 result in production of LPS with a single Hep attached. These mutants are able to synthesize Hep as indicated by the presence of attached Hep. A cloned gene capable of restoring expression of wild type LPS in a *S. typhimurium* LT2 mutant deficient in heptosyl transferase II has been isolated from *N. gonorrhoeae* (Schwan *et.al.*, 1995), *E. coli* (Schnaitman and Klena, 1993) and *C. jejuni* (J.Klena per. comm.).

The *waaQ* encodes heptosyl transferase III for third branch α(1-7) linked Hep to Hep II. The protein shows a clear region of homology with the *waa*C and *waa*F proteins (Sirisena *et.al.*, 1994; Yethon *et.al.*, 1998). To date, a *waaQ* homolog has not been characterised from *Campylobacter*. The third branch heptose as found in *E. coli* and *Salmonella spp.* appears to be absent in *C. jejuni*.

Phosphorylation of the inner core

Phosphorylation of KDO and Hep residues is considered to be an important feature for outer membrane permeability. The phosphoryl substituents in the heptose region are critical for ensuring outer membrane stability because the negative charge allows neighboring LPS molecules to be cross-linked by divalent cations. One gene of reported importance is *waaP*. Mutation analysis suggests that WaaP, by adding PPEA to Hep I, enables bacteria to keep the outer membrane impenetrable towards other hydrophobic compounds. Mutation of *waaP* resulted in loss of phosphoryl substituents on Hep I and obviated WaaQ and WaaY activity (Yethon *et.al.*, 1998). The *waaP* gene has been

cloned and sequenced from *E. coli* (Parker *et.al.*, 1992), *S. minnesota* (Jousimens and Makela, 1974) and *S. typhimurium.* The WaaY protein is another important phosphokinase that is involved in phosphorylation on Hep II. Phosphorylation of Hep II by WaaY requires transfer of Hep III (function of WaaQ) and phosphorylation of Hep I (Yethon *et.al.*, 1998).

1.6 ORGANISATION WITHIN LPS GENETIC LOCI

Hydropathy profiles suggest that few genes in the *waa* locus of *S. typhimurium* or *E. coli* encode membrane spanning proteins (Schnaitman and Klena, 1993). Gene products in this locus are almost exclusively soluble or membrane associated proteins, with no signal sequences typical of proteins translocated into the periplasm or outer membrane (Raetz, 1990). This supports the hypothesis that LPS is synthesized on the cytoplasmic face of the cytoplasmic membrane. Essential sugar nucleotides such as ATP and R-3-hydroxy-myristoyl-ACP are located in the cytoplasm.

In at least two members of the family *Enterobacteriaceae* most of the genes in the *waa* cluster are strongly conserved. The *waa* gene locus maps to 81 minutes on the *E. coli* chromosome and 79 minutes on the *S. typhimurium* chromosome (Bachman, 1990; Rocero and Casadaban, 1992). The cluster in *E. coli* K12 consists of a minimum of 17 genes while the *S. typhimurium* cluster has at least 13. The arrangement of the genes within the two loci appears to be highly conserved (Schnaitman *et.al.*, 1991; Schnaitman and Klena, 1993). Products of *waaBCDFGPQYZ* genes all exhibit 70% or more amino acid identity between *E. coli* and *S. typhimurium* in addition to their organisational conservation. *waaBCDFGQSZIJ* genes encode glycosyl, galactosyl or GlcN transferase. In general, the predicted amino acid sequences of genes located toward the centre of the *waa* gene locus appear less strongly conserved between genera (Schnaitman and Klena, 1993) or even within a species (Reeves *et.al.*, 1994a). This phenomenon in gene locus conservation may have arisen from a series of ancestral genetic exchanges involving very dissimilar organisms (Reeves *et.al.*, 1994a).

1.6.1 OUTER CORE DOMAIN

The outer core sugars glucose and galactose are sequentially attached to an α1,3-linked main chain Hep of LPS (Figure 3) to provide the structural framework for the outer, or hexose, region of the core (Schnaitman and Klena, 1993). The outer core region shows diversity with respect to the type of sugars present and the linkages by which they are joined. In *E. coli*, WaaGBORK, and *S. typhimurium*, WaaGBIJK, proteins are sugar transferases (Schnaitaman and Klena, 1993; Heinrichs *et.al.*, 1998). They share limited regions of sequence identity and appear to have a similar secondary structure. The galactosyltransferases are WaaI, which adds UDP-Gal to α(1-3)Glu II in *S. typhimurium*, and WaaB involed in attachment of UDP-Gal to α(1-6)Glu II in *E. coli* and *S. typhimurium*. Glucosyltransferases include *waaGO(I)R(J)*. The *waaG* product transfers the first outer core sugar, glucose I (UDP-glucose), to the inner core Hep II residue. *waaG* has been characterised in both *E. coli* (Parker, 1992) and *S. typhimurium* (Reeves *et.al.*, 1994b).

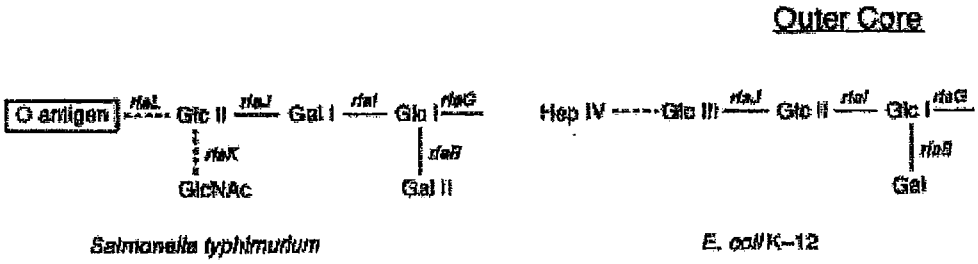


Figure 3. Simplified schematic overview of the outer core OS of *E. coli* K12, *S. typhimurium* LPS. Due to the heterogeneity in the chemical structures of LPS, the structures shown represent an average or consensus structure rather than a distinct molecular species.

In *C. jejuni*, galactose (linked β1-3) is the next addition on the chain after heptose II. However, D-glucose may instead be substitutied (Figure 4)(Aspinall *et.al.*, 1992). Other additions to the outer core and branches on the entire oligosaccharide appear to be variable in respect to the sequence of sugars, linkage types, anomeric configuration and degree of phosphorylation (Aspinall *et.al*, 1993a; Aspinall *et.al.*, 1993b). Unexpected

constituents such as sialic acid (N-acetyl neuraminic acid (Neu5Ac)) and 3-amino-3,6-dideoxy-D-glucose (Qui3N) have been located in some serotypes as branch or chain derivatives in the outer core (Aspinall *et.al.*, 1995). Neu5Ac is chemically related to KDO but is rarely found in procaryotes. When present it generally occurs as chain-linked polymer component of the capsular polysaccharide of *E. coli* K1 and K92 capsules, *N. meningitidis* (Vogel *et.al.*, 1997). *N. gonorrhoeae* (Mandrell *et.al.*, 1990), *Salmonella tourcra* (Kedzierska, 1978), *Rhizobium meliloti* (Defives *et.al.*, 1989) and *Rhodobacter* spp. (Krauss *et.al.*, 1988). Neu5Ac has low or no immunogenicity due to structural mimicry of mammalian host gangliosides. This may play a part in complement-mediated resistance (Moran *et.al.*, 1991a; Preston *et.al.*, 1996). The presence of Neu5Ac may cause increased resistance to human serum but the significance in *C. jejuni* pathogenesis in enteritis cases is uncertain (Salloway *et.al.*, 1996).

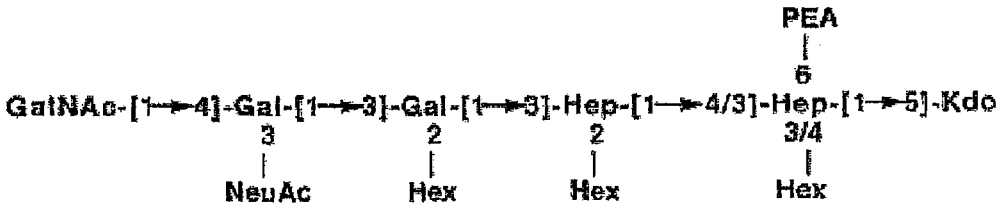


Figure 4. Strucure of the core OS from *C. jejuni* serotype HS:1

After addition of the last glucose in the core OS of *E. coli* K12 and *S. typhimurium* LT2, LPS structures differ. Genes encoding a glycosyl transferase responsible for addition of side chain saccharide moieties are thought to be *waaS* and *waaZ* in addition to *waaK* and *waaL*. The *waaK* product is an N-acetylglucosaminyltransferase involved in addition of a GlcNAc residue to the terminal core glucose in *S. typhimurium* (MacLachlan *et.al.*, 1991) and the inner LPS core in *E. coli* (Klena and Schnaitman, 1994). Attachment of GlcNAc (or glucose in *E. coli* K12) is a prerequirement for successful ligation of the O polysaccharide (Yethon *et.al.*, 1998). The ligase enzyme is envisioned as a glycosyltransferase, encoded by *waaL*, with a complex (lipid-linked oligosaccharide) substrate requirement (Reeves *et.al.*, 1994b; Whitfield, 1995). The role of WaaS and WaaZ is presently unknown, but their involvement in alteration of LPS is

clear. Data so far indicates that WaaS is involved in modification of O polysaccharide by adding TDP-Rhamnose to the nascent chain (Schnaitaman and Klena, 1993).

1.6.2 O ANTIGEN

The *rfb* gene locus maps to the 44-48 minute region in *S. typhimurium*, and to the 44 minute region of *E. coli* K12. This cluster contains genes that encode enzymes involved in O antigen subunit synthesis and polymerisation (Schnaitman and Klena, 1993). Biosynthesis of the O polymer occurs in association with the carrier lipid enzyme bactoprenyl pyrophosphate.

Detailed structural studies of the high M_rLPS from *C. jejuni* serotypes HS:19, HS:23 and HS:36 have demonstrated the presence of O-antigen chains that mirror O-antigens associated with "smooth" type LPS of *Salmonella* spp. (Mills *et.al.*, 1992). Each O polysaccharide contains repeating units in which two of the three residues (N-acetyl-glucosamine and galactose) are always identical but the third sugar residue is one of four similarly linked and stereochemically related Hep sugars (Mills *et.al.*, 1992). The point of attachment of the O-antigen side chain in *C. jejuni* is speculated to be in KDO or heptose region of the inner core, not the distal end of oligosaccharide as is the case with *Salmonella* spp. (Aspinall *et.al.*, 1994c).

1.7 OBJECTIVE OF THIS STUDY

Antigenic and phase variation caused by the *lex2B* gene in *H. influenzae* type b alters virulence (Jarosik and Hansen, 1994). An ORF downstream of *gmhA* in *C. coli* strain M275 was shown to have strong similarity with *lex2B* in *H. influenzae* (Upritchard, 1997). In *C. jejuni* strain NCTC11168 the presence of *lex2B* nucleotide sequence has not been detected, however a similar ORF is present in strain *C. jejuni* 928. The objective of this study has been to isolate and characterise *lex2B* from *C. jejuni* 928 and its potential role in pathogenesis of the organism. To accomplish this, identification of a functional *lex2B* gene in *C. jejuni* strain 928 and characterisation of both the gene and its product at a genetic and enzyme level as time permitted was to be performed.

Changes in carbohydrate composition and assembly of sugars in LPS results in increased cell surface diversity and leads to changes in virulence. The *lex2B* in *H. influenzae* and its analogs in other bacteria such as *H. somnus* (Inzana *et.al.*, 1997), *H. pylori* (Alm *et.al.*, 1999), *N. gonorrhoeae* (Erwin *et.al.*, 1996) and *N. meningitis* (Jennings *et.al.*, 1995) appears to have an important role in phase variation of LPS or LOS epitopes. The presence of the *lex2B* gene in LPS biosynthetic loci appears highly conserved in these bacteria. RFLP and sequence analyses have shown that the appearance of *lex2B* in *C. jejuni* and *C. coli* is an exception rather than the rule (Yates, 1998; Upritchard, 1997; Sanger centre, 1998). This suggests that *lex2B* is either a novel evolutionary addition to the *C. jejuni* and *C. coli* chromosome or a gene whose function is becoming non-essential for this genera. Furthermore the position of *lex2B* among LPS sugar transferases and the amino acid similarity between them suggests that *lex2B* could be a member of a glycosyl or galactosyl transferase super family.

The genus *Campylobacter* is not well characterised genetically. Successful molecular methodologies used to manipulate *E. coli* and *S. typhimurium* genetics have proven unsuccessful for *C. jejuni*. Interspecies complementation of *lex2B* in *E. coli* and *S. typhimurium* is not possible as these organisms lack this gene.

Investigation into the organisation and regulation of LPS biosynthetic genes in *C. jejuni* and construction of isogenic mutants should provide information on the role of LPS in *C. jejuni* virulence (Ketley, 1997). It may be possible to construct a live attenuated vaccine candidate, arising from the creation of isogenic mutants. This would be a mutant capable of causing an immune response in the host, yet unable to invade the host intestine.

1.7.1 AIMS

- To identify the location of the *lex2B* gene within *C. jejuni* strains 928 and NCTC11168.
- To determine whether the gene, if present, is expressed.
- To express the protein and develop a rapid test for *lex2B* detection.

- To create a null mutation to assess the role of the Lex2B protein in LPS biosynthesis, and subsequent pathogenesis.

These aims were carried out to determine if *lex2B* alters virulence of *C. jejuni*, by changes in LPS composition, as shown in other bacteria. By sequencing the *lex2B* gene from *C. jejuni* it will be shown whether the predicted Lex2B protein differs from that in *C. coli* M275, *H. pylori* and the non-enteric bacteria *H. influenzae*, *N. gonorhoeae*, *N. meningitis*. Sequence analysis was performed in order to assist in constructing a "null" mutation within the *C. jejuni* 928 chromosome and in prediction of possible connections that Lex2B has with other sugars transferases using hydrobic cluster analysis. Southern and dot-blot analyses were used to determine the copy number of *lex2B* on *C. jejuni* chromosome and detection of *lex2B* in other *C. jejuni* and *C. coli* strains. The *lex2B* gene expression was studied using RT-PCR to detect production of mRNA. Following expression analysis, expression of Lex2B protein was detected using the pET vector system for high-level protein expression. Purified protein was used to immunogenise rabbits to generate a polyclonal antibody. This antibody should provide an effective tool for screening all *Campylobacter* strains for the presence of the Lex2B protein.

This experimental procedure should provide strong evidence for *lex2B* expression and the possible functions of *lex2B C. jejuni* and *C. coli*.

CHAPTER II

MATERIAL AND METHODS

2.1 BACTERIAL STRAINS AND PLASMIDS

The *E. coli* strains used in this study are listed in Table 2.1. The *Campylobacter* strains used in this study are listed in Table 2.2. Plasmids used or developed in this study are described in Table 2.3.

Table 2.1. *E. coli* strains and plasmids used

Escherichia coli	**GENOTYPE**	**REFERENCE**
KLC 4000	DH5α; *Δlac*U169(*ϕ80lacZ* ΔM15) *lacYI, recA1*	Laboratory collection
KLC4157	χ705; F⁻*leu*-4ϕ ^{R}Str ^{R} *arg-35* T6^{R} λ⁻	Curtiss *et al.*, 1968
KLC4158	χ711; F⁻*leu*-4ϕ^{R} *ΔproAB118* T3^{R} *arg*-35 T6^{R}*ΔgmhA* Str^{R}	Curtiss *et al.*, 1968
KLC4166	F⁻ *dcm*⁻ *ompT*r$_{B}$ m$_{B}$⁻ *lon*⁻ λ (DE3) pLysS, Cm^{R}	Laboratory collection
KLC4171	KLC 4166//pET 11a, Cm^{R}, Ap^{R}	Laboratory collection
KLC4216	KLC4000//pDJ4216; *gmhA*$^{+}$, *lex2B*$^{+}$, Ap^{R}	This study
KLC4269	KLC4158//pDJ4216; *gmhA*$^{+}$, *lex2B*$^{+}$, Ap^{R}	This study
KLC4283	KLC4158//pGEM-T; *lacZ*', Ap^{R}	Laboratory collection
KLC4370	KLC4000//pJK4370; *lacZ*+, ApR, Km^{R}	Laboratory collection
KLC 4371	KLC4000//pJKP030; *Δlex2B*, Cm^{R}, Km^{R}	This study
KLC 4375	KLC 4000//pDJ4375; *Δlex2B*, Ap^{R}, Km^{R}	This study
KLC 4376	KLC4000//pMEK180; Km^{R}	Laboratory collection
KLC 4377	KLC 4166//pJKP030; *Δlex2B*, Cm^{R}, Km^{R}	This study

Abbreviations: Ap: ampicillin, Km: kanamycin, Cm: chloramphenicol, Str: streptomycin, Tet: tetracycline: ^{R}= resistant, :: = insert.

Bacterial strains followed by a double back slash (//) indicate that strain has been transformed with a plasmid.

Table 2.2. *Campylobacter* strains used

ISOLATE NAME	SOURCE	SPECIES
KLC4239	HF928 MedLab South	*C. jejuni*
NCTC11168(1958)	HF NZRM	*C. jejuni*
KLC4297	HF CHL	*C. jejuni*
KLC4303	HF CHL	*C. jejuni*
KLC4305	HF CHL	*C. jejuni*
KLC4315	HF CHL	*C. jejuni*
DO399V	HF CHL	*C. jejuni*
YP502F	HF CHL	*C. jejuni*
LG430P	HF CHL	*C. jejuni*
MB617S	HF CHL	*C. jejuni*
RC167B	HF CHL	*C. coli*
ZP028D	HF CHL	*C. jejuni*
M275	Dr. M. Konkel, Washington State University	*C. coli*

Abbreviations: HF: human faeces, NZRM: New Zealand Reference Material, CHL: Canterbury Health Laboratory

Table 2.3. Plasmids used in this study

PLASMID	DESCRIPTION	REFERENCE
pDJ 4216	pGEM-T::*gmhA*$^+$ *lex2B*$^+$ *ΔwaaF*, 4.7 kb, Ap^R	This study
pDJ 4269	pGEM-T::*gmhA*$^+$ *Δlex2B*, 4.1 kb, Ap^R	This study
pJK P010	pCR 2.1:: *Δlex2B*, 4.57 kb, Ap^R, Km^R	J. Klena
pJK P021	pCR 2.1::pMOMP:: *Δlex2B*, 4.85 kb, Ap^R, Km^R	J. Klena
pJK P030	pET24b:: *Δlex2B*, 5.95 kb, Km^R	J. Klena
pJK 3056	pBIISK+::*gmhA*$^+$ *lex2B*$^+$ *waaF*$^+$, 5.8 kb, Ap^R	J. Klena
pJK4370	pBIISK+:: Km200, 4.26 kb, Ap^R, Km^R	J.Klena
pMEK180	*Campy ori*, 10 kb, Km^R	J. Klena
pDJ4375	pJK4370:: *Δlex2B*, 4.9 kb, Ap^R, Km^R	This study
pBluescript II SK+	*lacZ'*, ColE1*ori*, 2.96 kb, Ap^R	Stratagene
pGEM-T	*lacZ'*, ColE*ori*, 3.0 kb, Ap^R	Promega
pET24b	*lacI'*, f1*ori*, 5.3 kb, Km^R	Stratagene
pCR 2.1	*lacZ'*, ColE1*ori*, 3.9 kb, Ap^R, Km^R	Invitrogen®
pET11a	*lacI*^{q}, ColE1*ori*, 5.6 kb, Ap^R	Stratagene

2.2 BUFFERS AND MEDIA

Solutions and media used in this study were prepared as described in Appendices 1 and 2.

2.3 BACTERIOLOGICAL METHODS

2.3.1 CULTURE CONDITIONS

C. jejuni and *C. coli* isolates were incubated in a water-jacketed CO_2 incubator (Nuaire) in a microaerophilic environment (10% CO_2), at 37°C on solid media (*Campylobacter* charcoal differentiation agar, (CCDA, Oxoid)) with the addition of cefoperazone (Cp) or on Mueller-Hinton medium. The concentrations of antibiotics used in this study are listed in Table 2.4.

All *E. coli* strains were incubated aerobically at 37°C in Luria Bertani broth (LB) or LB broth containing 1.5% agar (LBA). Overnight cultures were inoculated from a single, isolated colony taken from a streak plate. Antibiotics and colourimetric reagents were added to liquid or solid media when necessary, to maintain selection pressure or to identify constructs, as indicated in Table 2.4.

Table 2.4. Antibiotics and supplements used in this study

ANTIBIOTIC/CHEMICAL	ABBREVIATION	FINAL CONCENTRATION
Ampicillin	Ap	50μg/ml
Chloramphenicol	Cm	20μg/ml
Kanamycin	Km	30μg/ml
Tetracycline	Tet	15μg/ml
Cefoperazone	Cp	32 μg/ml
5-bromo-4-chloro-3-indoyl-β - D-galactopyranoside	X-gal	40μg/ml
Isopropyl-β-D-thiogalactoside	IPTG	25 mg/ml

2.3.2 STORAGE OF STRAINS

For day to day use *Campylobacter* strains were maintain on CCDA plates supplemented with Cp. *Campylobacter* isolates were passaged every 48 h to maintain culturability. Long term storage of isolates was achieved by harvesting bacterial growth from CCDA

plates using Brain Heart Infusion broth (Gibco BRL) containing sterile glycerol (BDH) (final concentration of 20%) and sterile 6mm glass beads (BDH) contained in Nunc cryotubes. Aliquots of isolates were stored at –80°C until required.

E. coli isolates were maintained on LBA streak plates, supplemented with antibiotics as required, at 4°C. For long term storage strains were maintained in 20% glycerol (final concentration) at –80°C.

2.4 DNA MANIPULATION AND CLONING TECHNIQUES

2.4.1 PREPARATION OF TOTAL GENOMIC DNA

Isolation of chromosomal DNA from *Campylobacter spp.* was performed using the method of Pospeich and Neumann (1995). Briefly, 5ml of cold, sterile ddH_2O was used to harvest *Campylobacter* growth from plates as described (see section 2.3.1). Cells were pelleted by centrifugation at 3700×g for 10 min at 4°C. Cell pellets were resuspended in 250µl of SET buffer (Appendix 1) containing lysozyme (final concentration 1mg/ml) and incubated at 37°C for 60 min. 1/10 volume of 10% SDS and proteinase K (final concentration 0.5mg/ml) were added and cell suspensions incubated at 55°C for 2 h (or until lysis was complete), with occasional inversion. After completion of lysis, 1/3 volume of 5M NaCl and 1 volume of chloroform were added and incubated, with frequent inversion, at ambient temperature for 30 min. Mixtures were centrifuged (14800×g for 15 min) and aqueous phases were transferred to sterile tubes. An equal volume of phenol/chloroform/isoamyl alcohol (IAA) was added to cell extracts, mixed via inversion, centrifuged as above and extracted. Chromosomal DNA was precipitated by adding 2 volumes of absolute ethanol and 1/10 volume of 3M sodium acetate. Genomic DNA was collected by centrifugation for 15 min at 17400×g, washed with 70% ethanol and air-dried. DNA was dissolved in 100µl of sterile ddH_2O containing *Rnase*A (final concentration of 20µg/ml).

2.4.2 PLASMID EXTRACTION

Small scale plasmid isolations were performed using the lysis by alkali method (Sambrook *et.al.*, 1989). Plasmid DNA to be further manipulated was purified from excess salts or other contaminating particles by one of two methods. The first method used the GeneClean III kit (Bio101, La Jolla, CA) according to the manufacturer's protocol.

The second method was used to salt precipitate plasmid DNA that was to be further manipulated (e.g. restriction enzyme digested, ligated, and/or to have its nucleotide sequence determined). This method involved addition of an equal volume of phenol/chloroform/isoamyl alcohol (IAA) to the resuspended DNA. The two solutions were thoroughly mixed and the organic and aqueous phases separated by centrifugation at 17400×g for 4 min. The aqueous phase was transferred to a 1.5 ml Eppendorf tube and two volumes of absolute ethanol were added, mixed and incubated at ambient temperature for 5 min. DNA was harvested by centrifugation (17400×g, 10 min, 4°C), washed with 1 ml of 70% ethanol and air dried before resuspending the DNA in 100µl ddH_2O.

2.4.3 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digestions

In general, restriction endonuclease digestion reactions were carried out in 15µl volumes, according to the manufacturer's recommendations. Digestions were usually incubated at 37°C for 2-3 h unless otherwise specified. Digestions involving two enzymes were performed using the most suitable enzyme buffer or by addition of NaCl. However when this was not possible, DNA was digested initially with one endonuclease using the most appropriate enzyme buffer, then salt and enzyme were removed by phenol/chloroform/IAA precipitation, and DNA digested with a second endonuclease using the most appropriate buffer.

Dephosphorylation of 5' overhangs

To prevent intramolecular (self-ligation) of vector or insert DNA, calf intestinal alkaline phosphatase (CIAP) was added to the restriction endonuclease digestion mixtures after complete digestion. CIAP removes 5' phosphate overhangs from DNA. The digestion mixture, 2µl of 10× dephosphorylation buffer (Gibco BRL), and 1 unit of CIAP (Gibco BRL) were brought up to a final volume of 20µl and incubated at 37°C for 30 min. The reaction was terminated by addition of 5mM EDTA (pH 8.0) and incubated at 65°C for 20 min. The reaction volume was adjusted to a total of 100µl and the mixture was extracted once with phenol:chloroform:IAA to remove enzymes. DNA was precipitated by addition of 1/3 volume 3 M sodium acetate (pH 4.8) and 2 volumes of cold 100% ethanol. After incubation at room temperature for 5 min, the mix was centrifuged at 17400×g for 10 min, washed with 70% ethanol and the pellet air dried briefly before resuspension in 20 µl dH_2O.

2.4.4 AGAROSE GEL ELECTROPHORESIS OF DNA

Gel electrophoresis

Restriction endonuclease digested DNA fragments were separated by electrophoresis through agarose (0.8%-2%) using Tris-Acetate-EDTA (TAE) buffer (Appendix1). Reactions were mixed with 1-2 µl 6×bromophenol blue gel dye (Appendix 1) before loading into agarose gels. DNA fragments were separated by exposure to an electrical current for one hour at 100V, in a Biorad mini sub or DNA sub cell apparatus. Gels were stained in water containing ethidium bromide (0.5 µg/ml) for 20 min. DNA was visualised using a Sigma T2210 UV (302 nm) transilluminator or an Ultra-Lum KS-3000 visualisation-documentation and analysis system (Ultra-Lum, Carson, CA).

Elution of DNA fragments from agarose gels

After electrophoresis and staining, digested DNA fragments were excised from agarose using a sterile scalpel blade and placed into sterile 1.5 ml Eppendorf tubes. DNA was

extracted from agarose by using the GeneClean III (Bio 101, La Jolla, CA) or Prep-a-Gene kits (Biorad), according to methods supplied by the manufacturers. DNA was resuspended in ddH_2O.

2.4.5 DNA LIGATION

DNA from various sources were ligated together using either 1 µl of T4 DNA ligase (Gibco BRL) and 4 µl of 5× DNA ligase buffer (Gibco BRL) in a final volume of 20 µl or 1µl of T4 ligase (Promega) and 1µl of 10× DNA ligase buffer in a final volume of 10 µl. Ligation mixtures were incubated at 12°C for 12-24 h. Ligation mixes were either kept on ice or at –20°C until use. A vector:insert ratio of 1:3 was favored for high intermolecular ligation and low concatamer formation.

2.5 PLASMID CONSTRUCTS

Plasmids were constructed for use in complementation analysis, as substrates for mutagenesis, Southern hybridisation, DNA sequence determination and protein expression analysis. In this section I describe the important plasmids that have been constructed for use in this study.

pDJ 4216

A 1.7 kb PCR amplicon was generated using the primers 96.01 and 94.293 with DNA from 928, (*C. jejuni* KLC4239) as template. The amplicon was cloned into the modified *Eco*RV site (overhanging poly – T's) of pGEM-T (see Table 2.3). This plasmid construct containes *gmhA*$^+$-*lex2B*$^+$-*waaF'* region.

pDJ4375

pJKP010 was restriction digested with *Eco*RI and *Bgl*II. A 0.64 kb *Eco*RI fragment was excised from the agarose gel, prepared as above (section 2.4.4) and ligated into pJK4370 digested with *Eco*RI. The resulting plasmid contained *lex2B* without 5' and 3' ends and

also a Km-resistance gene. As this plasmid would encode for a Lex2B protein deficient in N and C termini, this plasmid construct was used to create "null" mutation, by single cross homologous recombination, on *C. jejuni* 928 chromosome.

pJKP030

A 0.642 kb PCR amplicon was generated using primers 99.23 and 99.25 with pDJ4216 (*C. jejuni* 928 DNA) as the template. Two restriction enzyme sites were created, one in the forward 99.23 primer (*Sst*I) and one in the reverse primer 99.25 (*Xho*I). The 640 bp amplicon was digested with the restriction enzymes, *Sst*I and *Xho*I, and ligated into the expression vector pET24b (see Table 2.3), also digested with the same enzymes. The resulting plasmid, designated pJKP030, was transformed into KLC4000, reisolated and digested again to verify its structure. This plasmid construct was used in expression analysis of Lex2B in a T7 RNA polymerase-based gene expression system.

2.6 TECHNIQUES FOR STRAIN CONSTRUCTION

2.6.1 CALCIUM CHLORIDE TRANSFORMATION

E. coli strains were transformed with plasmid DNA constructs using one of two methods. The first method involved preparing cells with $CaCl_2$. Competent cells were prepared using a variation of the Sambrook *et al.* (1989) method. The $CaCl_2$ method is rapid and easy to perform. This method is summarised as follows: a culture grown overnight in LB broth at 37°C was diluted 1:100 into 10 ml of LB broth and incubated with aeration at 37°C to mid-log phase (generally 2h without shaking and 1h shaking at 225 rpm in a water bath). Bacterial cells were pelleted by centrifugation (10 min at 4°C), resuspended in 0.5 volume of cold 100 mM $CaCl_2$ and incubated on ice for 60 min. Cells were pelleted by centrifugation (10 min at 4°C), resuspended in 0.08 volume of ice cold 100 mM $CaCl_2$ and incubated on ice or 60-90 min prior to transformation. At this point the competent cells could be stored overnight at 4°C, or at –80°C for several months by addition of sterile glycerol to a final concentration of 15-20%.

For each experiment 100-200µl of $CaCl_2$-competent cells was used. To these cells 0.05-0.3µg of plasmid DNA (usually about 5µl of plasmid DNA from the alkaline lysis procedure) was added and the mixture incubated on ice for 30 min. Mixtures were heat-shocked at 42°C for 90 sec without shaking and placed immediately on ice for 2 min. Transformants were diluted in 800 µl LB broth and grown at 37°C for 1h to allow for expression of antibiotic resistance genes. Transformed cells were harvested in a tabletop centrifuge for 1 min and resuspended in 200µl LB broth. Transformants were spread-plated onto LBA supplemented with appropriate antibiotics and cultured overnight at 37°C unless otherwise specified. Single transformants were purified and plasmid DNA was isolated to verify the presence of the correct construct.

2.6.2 *Campylobacter jejuni* COMPETENT CELLS

Campylobacter spp. are naturally competent for acquisition of homologous DNA, however for heterologous and plasmid DNA competence drops significantly. *C. jejuni* cells that have been made electrocompetent are more susceptible for transfomation with plasmid DNA.

The method for making *C. jejuni* competent cells (M. Konkel per. comm.) is summarised as follows: a *C. jejuni* culture was grown on Mueller-Hinton agar with blood (MHBA) using conditions described in section 2.3.1. Bacterial growth was harvested from a plate using 5 ml of Mueller-Hinton broth without blood and transfered into 250 ml of the same medium. Cultures were incubated in a sealable jar with a candle to create a microaerophilic environment. After 24h (or until reaching approximately 1×10^7 cfu/ml), cells were chilled on ice for 20 min. Bacterial cells were pelleted by centrifugation at 5150×g for 10 min at 4°C. The resulting pellet was resuspended in 250ml of ice cold, sterile water. Cells were pelleted as before and resuspended in 125ml of ice cold ddH_2O. Cells were harvested as above and resuspended in 5ml of ice cold sterile 10% glycerol. In final step, cells were pelleted as before, resuspended in 0.5 ml of ice cold sterile 10% glycerol and aliquoted into 50 µl aliquots. Electroporation of the plasmid was carried out as described in section 2.6.3. After electroporation bacterial cells were resuspended in 200µl of Mueller-Hinton broth and plated on MHBA without

antibiotic. Following 24 h incubation as described in section 2.3.1, *C. jejuni* growth from the non-selective plates were streaked onto MHBA with kanamycin (final concentration 200 μg/ml). Transformants were expected within 48 h, and spontaneous revertants beyond this period.

2.6.3 ELECTROPORATION

Electroporation has been demonstrated to be a more efficient means of DNA transfer than $CaCl_2$ transformation. Competent cells were prepared using a variation of the protocol supplied with the Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA). An overnight culture was diluted 1:100 into LB broth and incubated until early mid-log phase (typically 2 h without shaking and 1h with shaking at 37°C) and chilled on ice for 20 min. The cells were centrifuged at 10300×g for 10 min at 4°C. The cell pellet was resuspended in 1 volume of deionised water (dH_2O) and collected as before. The pellet was then resuspended in 0.5 volume dH_2O and cells collected as before. The pellet was resuspended in 0.08 volume of cold, sterile 10% glycerol, transferred to a 1.5 ml Eppendorf tube and centrifuged at 10300×g for 10 min at 4°C. The cells were resuspended in 0.0032 volume of sterile 10% glycerol and divided into 40 μl aliquots for immediate use, or stored at –80°C for several months. Electroporation was carried out using a Gene Pulser™(Bio-Rad) with the following settings: 25μF, 2.5kV and 250ohms. Electrocompetent cells were mixed with 3 μl of plasmid DNA (usually 0.05-1.0μg) for one min before the mix was transferred to an ice cold gene pulser cuvette (Bio-Rad) and pulsed at the above settings, usually giving a time constant of 4.0-4.6 msec. Immediately following the pulse, 1ml of SOC (Appendix 1) was added to the transformants, these were transferred to a sterile Eppendorf tube and incubated at 37°C for 1h with aeration. Transformants were pelleted by centrifugation for 1 min at 17400×g and resuspended using 100 μl of fresh LB broth and spread plated onto selective LBA plates.

2.7 RNA MANIPULATION

2.7.1 ISOLATION OF TOTAL RNA

5 ml of cold sterile ddH2O was used to harvest *Campylobacter* growth from a plate incubated as described (see section 2.3.1). 1 ml (~1×10^9 cells) of this suspension was centrifuged at 5000×g for 5 min at 4°C. The resultant pellet was resuspended with lysozyme-containing TE buffer (final concentration of lysozyme- 400µg/ml) and incubated for 10 min at ambient temperature. RNA was isolated using the protocol supplied within the RNeasy Mini Kit (Qiagen). After isolation, total RNA was digested with *DNase*I (3U/µl) (Gibco BRL)

2.7.2 HANDLING RNA

Precautions to avoid introducing RNases into the RNA sample during or after the isolation procedure were performed. Glassware, electrophoretic tanks and solutions used for RNA work were treated with 0.1% diethyl pyrocarbonate (DEPC). Proper microbiological, aseptic techniques for RNA work such as use of aerosol barrier tips and frequent changes of gloves were applied. Work was done in a specially adapted RNA cabinet constructed of Perspex and containing a UV lamp.

2.7.3 RT-PCR

Detection of mRNA by the two step Reverse Transcription-PCR (RT-PCR) was chosen to study the expression of *lex2B*. Prior to first strand synthesis, total RNA (1-5µg) was divided into the three Eppendorf tubes (10µl of RNA into each tube). One tube was digested with 3U/µl *DNaseI* (GibcoBRL) for 25 min at 20°C. A second tube was treated with 0.5 µl of 10mg/ml *RNaseA* for 30 min at 37°C and a third sample was digested with a combination of these enzymes. *DNaseI* was inactivate by heating at 95°C for 5 min. All buffers and enzymes were purchased from Gibco BRL.

The first step, synthesis of cDNA, was carried out in a 20µl reaction volume containing 1µl of total digested RNA. RNA was mixed with 4µl 5× First strand buffer, 2µl 0.1M

DTT, and 10mM dNTP mix and incubated for 2 min at 42°C. Superscript™ II (reverse transcriptase) was added and samples were incubated at 42°C for 50 min. Reactions were stopped by incubation at 70°C for 15 min. After cDNA synthesis, additional digestion with *RNaseA* was performed on some samples to remove RNA complementary to cDNA.

In a second step, 10% of the first strand reaction was used for PCR. Samples were amplified using the two sets of primers: 98.21 (Forward)/ 94.375 (Reverse) as *lex2B* internal primers and 97.21 (Forward)/97.23 (Reverse) 16S rDNA primers as a positive control (see Table 2.5). PCR amplification was initiated with a three stage programme consisting of 32 repeated cycles (see section 2.10).

2.8 EXPRESSION OF *lex2b* IN *E. coli*

A bacteriophage T7 RNA polymerase/promotor system developed by Studier and Moffatt (1986) was used to study expression of *lex2B* in *E. coli*. This system allows high levels of expression of some genes that are not expressed efficiently in other systems or produce a product toxic to the *E. coli* cells.

2.8.1 INDUCTION OF *lex2B* IN pET24b CELLS GROWN IN STRAIN KLC4377 *E. coli* UNDER T7 PROMOTER CONTROL

For induction of the *lex2B* encoded protein from *C. jejuni* 928 strain into *E. coli* KLC4377, methods recommended by the manufacturer (Stratagene) of the pET vector system were used. Briefly, KLC4166, KLC4171 and KLC4377 were grown from single, isolated colonies in 10 ml of LB medium containing Ap and Cm at 37°C for 16 h. 2 ml of these cultures were used to inoculate 50 ml of fresh LB broth containing selective antibiotics and incubated at 37°C with aeration. Cultures were grown until an OD_{600} of 0.4-0.6 was reached. 1ml aliquots of each culture was removed, to serve as an uninduced control. IPTG was added to the remaining cultures from a 500mM stock to a final concentration of 2mM. Incubation was continued for 24h, measuring OD_{600} every 30 min for 5h, with subsequental removal of 1ml aliquots. The last sampling was carried

out after 24h. Immediately after removing 1 ml of culture from each reading, samples were centrifuged at 10300×g for 5 min, the pellet was resuspended in appropriate volume of ddH_2O and 1 volume of 2× loading buffer (Appendix II).

In order to visualise induction of the target protein Lex2B, samples were boiled for 5 min and analyzed by Commassie Brilliant Blue staining of an SDS-PAGE gel (25 µl loaded sample loaded into a 15% separating polyacrylamide gel).

2.8.2 QUANTITATION OF PROTEINS - BRADFORD METHOD

The Bradford method is a rapid and easy method to quantitate proteins. This method depends on quantitification of a dye, Coomassie Brilliant Blue, binding to unknown proteins and comparing this binding to that of different known concentrations of a standard protein, such as bovine serum albumin (BSA).This method was used to quantitate the amount of protein present after each sampling in protein expression studies. Briefly, 60 ml of each sample was mixed with 3 ml of Bradford solution which contains Commasie Brilliant Dlue dye (Protein Assey Kit, BioRad). Mixtures were gently inverted, vortexed and left at ambient temperature for 15 min. Samples were transferred into disposible cuvettes (BioRad) with path length 1 cm. Concentration of proteins in samples at A_{600} was determined using Hitachi U-200 spectrophotomer.

2.9 LIPOPOLYSACCHARIDE ANALYSIS

2.9.1 ISOLATION OF BACTERIAL LIPOPOLYSACCHARIDE

Bacterial lipopolysaccharide was isolated using a variation of the method described by Hitchcock and Brown (1983). Briefly, 5 ml of sterile ddH_2O was added to *Campylobacter* growth as described in section 2.3.1. All growth was scraped and 1.5 ml was transferred to an Eppendorf tube. Cultures were centrifuged at 17400×g for 5 min. Pellets were resuspended in 200µl of lysis buffer (20% glycerol, 5% 2-β-mercaptoethanol, 4.6% SDS, 0.125 M Tris-hydrochloride-buffered saline (pH 6.8), and 0.004% bromophenol blue) and boiled for 5 min. Samples were allowed to cool for 5

min before incubation at 37°C for 1h in the presence of proteinase K (final concentration of 0.4 μg/μl). After incubation, samples were cooled on ice and briefly microfuged, and diluted 1:2 with 60 ml of lysis buffer without proteinase K before freezing at -20°C. Prior to electrophoresis, LPS samples were boiled for 5 min and diluted 1:10 in lysis buffer. 5 μl of each sample was loaded into a designated lane in SDS-PAGE gel comprised of 4.5% stacking gel and 18% separating polyacrylamide gel.

E. coli LPS was isolated by the same method except 0.5 ml of an overnight culture was used as starting material and the composition of the lysis buffer was altered (10% glycerol, 1 M Tris (pH 6.8), 4% 2-β-mercaptoethanol and 0.05% bromophenol blue).

2.9.2 LPS ELECTROPHORESIS

LPS fragments were separated using sodium dodecyl sulfate (SDS)-tricine buffer system (top and bottom buffer are described in Appendix 2) described by Lesse *et al.* (1990). Polymerisation was catalysed with TEMED initiation using 10% ammonium persulfate (APS). A Biorad Protean™ III gel apparatus (Biorad) was used in casting and electrophoresis of samples (dimensions 10 × 10 with 0.75 mm spacer) with a Pharmacia Biotech EPS 600 power supply set at constant current (35 mA). When electrophoresis was completed (i.e. when the bromphenol blue dye front reached the bottom of the gel), the plates were separated and the SDS-PAGE gel was fixed (25% v/v isopropanol and 7% v/v acetic acid) for 16h.

2.9.3 SILVER STAINING OF LPS SDS-PAGE GELS

LPS was visualised using a modification of the silver staining method of Hitchcock and Brown (1983). Briefly, fixed gels were oxidised in freshly made solution one (Appendix II) for 5 min followed by eight ddH_2O washes (2×30 min, 2×20 min, 4× 10 min) in 200 ml. The water was aspirated off and 150 ml of freshly made silver stain (Appendix 2) was used to stain the gel for 10 min. Silver stain was decanted and the gel was washed four times for 10 min in 200 ml of ddH_2O. The gel was developed in 200 ml of citric acid developer (Appendix II) for 1-20 min. Stop solution (200 ml ddH_2O, 10 ml 7% v/v

acetic acid) was added when LPS and background were highest contrast. In all steps, gels were agitated on an orbital shaker set at a slow speed. Gels were normally allowed to soak for a minimum of one hour and photographed using a SLR camera with colour slide photography.

2.10 SOUTHERN HYBRIDISATION

2.10.1 SOUTHERN TRANSFER

Genomic DNA was digested to completion using an excess of a restriction enzyme (e.g. *HinD* III) and separated by electrophoresis through an 1% agarose gel in a Bio-Rad mini sub cell apparatus. DNA located in the gels was stained using water containing ethidium bromide (0.5 μg/ml) and photographed with Polaroid 667 film (ASA 3000). DNA was transferred to a 10×15 cm positively charged nylon membrane (Roche) using a Pharmacia LKB VacuGene XL vacuum blotting system (Pharmacia) and 20×SSC for 90 min as recommended by the manufacturer. After this period the gel was restained with ethidium bromide and DNA visualised using an UV transilluminator to determine the efficiency of transfer. The DNA was fixed to the membrane by crosslinking for 30 sec at 1200 KJ in an U.V.C-515 ultraviolet multilinker (Ultra-lum). Membranes were either probed immediately or wrapped in foil and stored at ambient temperature until required.

2.10.2 PROBE LABELING, HYBRIDISATION AND SIGNAL DETECTION

The non-radioactive digoxygenin-dUTP (DIG) DNA labeling and detection kit (Roche) was used for detection of all Southern hybridisations. Hybridisation was performed according to the procedure described in the instruction manual. Briefly, the membrane was placed in a Hybaid glass tube, prewarmed at 42°C with 10 ml of hybridisation buffer (DIG EasyHyb, Roche). Prehybridisation was carried out for 2 h at 42°C in Hybaid LTD Micro-4 HB-MCR4 oven. Prehybridisation solutions were discarded and replaced with 10 ml of hybridisation solution containing denatured DIG-labeled probe (final concentration 25ng/ml). DIG-labelled probes were denatured (10 min at 100°C)

prior to addition in hybridisation buffer. Hybridisation was performed at 42°C for 16 h with constant rotation.

Following hybridisation membranes were washed twice (15 min per wash) in low stringency wash buffer (2 × SSC, 0.1 % SDS) at ambient temperature to remove unbound probe. Membranes were washed further (twice for 15 min) in high stringency buffer (0.5 × SSC, 0.1 % SDS) at 68°C with constant rotation. Hybridised DNA was detected as described by the manufacturer. Membranes were sealed in plastic with approximately 1 ml of CSPD and incubated at 37°C for 15 min. Results were documented by photography.

2.10.3 DOT-BLOT HYBRIDISATION

To determine if the *lex2B* gene was present on chromosome of other *C. jejuni* and *C. coli* isolates, dot-blot analysis was performed with a portion of the *lex2B* gene from *C. jejuni* 928 which has been DIG-labelled (see section 2.10.2). This fragment was also used in Southern hybridisation experiment (section 2.10.2) To a positively charged membrane (Roche) chromosomal DNA was added with final concentration of 10 ng/µl. The DNA was UV cross-linked to the membrane and developed as described in section 2.10.2.

2.10.4 STRIPPING MEMBRANES FOR REPROBING

An advantage of the DIG detection system is that both the membrane and the probe are reusable. After hybridisation, remaining hybridisation solution containing DIG- labelled probe was stored at –20°C. Before reuse the hybridisation solution containing DIG-labelled probe was freshly denatured at 68°C for 10 min.

Membranes were stripped by rinsing in ddH_2O followed by incubation at 37°C in probe stripping solution (0.2 N NaOH, 0.1 % SDS) for 30 min. Membranes were rinsed in 2×SSC before reprobing.

2.11 POLYMERASE CHAIN REACTION OF PLASMID AND GENOMIC DNA

Polymerase chain reaction (PCR) of DNA (genomic and plasmid) and RNA was performed on a Corbett Research FTS-320 Thermal Sequencer. Primers for PCR were purchased from Gibco BRL. Deoxynucleotides (dNTPs), buffers and enzymes were purchased from Roche.

In general, approximately 100 ng/ml of DNA was used for each reaction. Each PCR consisted of several reagents added to a thin-walled 0.5 ml Eppendorf tube (all concentrations are final for 100 µl reactions): 3 pmol/ml of each primer (forward and reverse), 250µM dNTPs (dATP, dTTP, dCTP, dGTP), 1.5mM $MgCl_2$ and sterile ddH_2O. To this mix was added 10 µl of 10× PCR buffer, 1 µl of plasmid DNA and 0.5 µl of *Taq* DNA polymerase. Mixtures were briefly pulsed in the centrifuge to collect all components of the reaction. Reaction mixtures were overlaid with 100 µl of sterile mineral oil and placed in the thermal sequencer. PCR amplification was initiated with a three stage programme of 32 repeated cycles. Each cycle consisted of a denaturation step (94°C for 1 min), an annealing step (55°C for 1 min) and an extension step (72°C for 1 min). The annealing temperature was based on the Tm value of the primer (Bolton and McCarthy equation; Sambrook, 1989) and PCR products of 1 kb or larger were given an increased extension time. A final cycle used an extension time of 5 min to ensure that all PCR products initiated were able to be completed. PCR products were incubated at 4°C until removed from under the paraffin oil and stored at -20°C. Control reactions containing all reagents except template, absence of one primer or *Taq* polymerase were performed with every new primer or template tested.

Table 2.5. PCR primers

PRIMER	PRIMER NUCLEOTIDE SEQUENCE	TARGET
97.01 (Forward)	5'-GAA TGG CAA GAA CAT CA-3'	*gmhA*
SP6 (Reverse)	5'-ATT TAG GTG ACA CTA TAG-3'	Sp6 promoter, e.g. pGEM-T
T3 (Forward)	5'-AATTAACCCTCACTAAAGGG-3'	T3 promoter, e.g. pBIISK+
T7 (Forward)	5'-TAA TAC GAC TCA CTA TAG GG-3'	T7promoter, e.g. pBIISK+, pGEM-T
98.21 (Forward)	5'-AAT GAA AGT TTT TAT CAT-3'	*lex2B*
94.375 (Reverse)	5'-CCT TGA GTT CCT GCA AG-3'	*lex2B*
94.293 (Reverse)	5'-GCA ATA AAA CCA CTA TCA-3'	*waaF*
97.21 (Forward)	5'-AGA GTT TGA TGM TGG CTC AG-3'	16S rDNA (*E. coli*)
97.23 (Reverse)	5'-GWA TTA CCG CGG CKG CTG-3'	16S rDNA(*E. coli*)
99.23 (Forward)	5'-GAG CTC AGA GCG TTC TTT AGA TAG G-3'	*lex2B*
99.25(Reverse)	5'-CTC GAG TTT TCT TCC ATA TTC TAC AAT AAC AC-3'	*lex2B*
*gmhD*DV	5'-GCT CAT TTG GTA GGA CTT AG-3'	*gmhD*

Abbreviations: M= A or C; K= G or T; W= A or T;
red indicates generated restriction enzyme sequences.

2.12 SEQUENCING

DNA sequencing is the determination of the deoxynucleotide order of a specific segment of DNA. In this study DNA sequences were deduced using the chain termination method. The chain termination method or "Sanger dideoxy sequencing", depends on the enzymatic synthesis of labeled DNA, using modified nucleotides to terminate elongation of newly synthesised DNA (Sanger *et al.* 1977).

DNA sequencing was achieved using an ABI Prism 377 DNA sequencer and fluorescent dye terminator technology at Waikato University.

CHAPTER III

RESULTS

3.1 FUNCTIONAL IDENTIFICATION OF *C. jejuni* 928 *gmhA-lex2B-waaF* GENES

Previous analyses revealed the presence of a novel arrangement of genes involved in heptose synthesis within *C. coli* M275 (Upritchard, 1997; Yates 1998). This arrangement, *gmhA-lex2B-waaF*, was not seen in the *C. jejuni* NCTC11168 sequence strain (genome completely sequenced- Sanger, 1998). However, *C. jejuni* strain 928 was shown to produce a PCR amplicon of 1.7 kb when primed with primers 96.01 and 94.293 (Yates, 1998). This is a PCR amplicon equivalent in size to that seen from *C. coli* M275. In 71.4% (n=105) of *C. jejuni* isolates from human infections (Yates, 1998), including NCTC11168, these primers generate a 0.9 kb PCR amplicon. The purpose of this thesis was to characterise the *lex2B* gene and to determine if it is present in some, but not all, *C. jejuni* isolates. Thus the first objective was characterisation of the 1.7 kb PCR amplicon from *C. jejuni* 928.

3.1.1 A PLASMID CONTAINING *gmhA-lex2B-waaF'* GENES FROM *C. jejuni* 928

In order to characterise the 1.7 kb PCR amplicon generated from *C. jejuni* 928, restriction digestion analysis was performed. Cleavage sites for the restriction enzymes *Eco*RI and *Pst*I were observed to occur in the 1.7 kb fragment of M275. Digestion of the 1.7 kb PCR amplicon from 928 with these enzymes showed the same restriction enzyme profile as *C. coli* M275 (data not shown). This PCR amplicon was cloned into the expression plasmid pGEM-T (see section 2.5) resulting in new plasmid construct, pDJ4216. If the cloned insert was the fragment of DNA expected to be amplified, a fully functional *gmhA* gene should be present.

3.1.2 *gmhA* CROSS-SPECIES COMPLEMENTATION

In order to test whether pDJ4216 contained a functional *gmhA* gene, transformation of an *E. coli gmhA*$^-$ strain with pDJ4216 plasmid, was tested. Restoration of WT LPS would suggest the presence of *gmhA* activity on pDJ4216.

For functional analysis of *gmhA* from *C. jejuni* 928, *E. coli* K12 strain KLC4158 was used. KLC4158 has a 35 kb IS5-mediated deletion spanning the *gmhA* locus resulting in the inability to produce the glyceromannophosphate isomerase enzyme (see Table 2.1). Due to this defect in heptose biosynthesis, KLC4158 produces truncated LPS and as a result, is supersensitive to hydrophobic antibiotics and bile salts. KLC4158 was transformed with pDJ4216 to determine if the 1.7 kb fragment from *C. jejuni* 928 could restore to KLC4158 the ability to grow on MacConkey bile salts rich medium and restore full length LPS.

Following electroporation, a total of seven transformants were harvested and patch streaked onto LBA, LBA+Ap and MacConkey media together with the control strains KLC4157 (*gmhA*$^+$) and KLC4158 (*gmhA*$^-$). All seven KLC4158 transformants as well as KLC4157 grew on MacConkey media, while strains KLC4158 and KLC4283 (KLC4158 harbouring pGEM-T) failed to grow in this bile salts rich medium. Plasmid DNA was extracted from the KLC4158 transformants and restriction digested with *Pst* I and *Eco*RI. Resultant fragments were of a size predicted from restriction digestion of the PCR amplicon of 1.7 kb amplicon of *C. jejuni* 928 (data not shown).

The above evidence suggested glyceromannophosphate isomerase activity from *C. jejuni* 928 is present and functional as judged by restoration of heptose biosynthesis on pDJ4216. Following these results, nucleotide sequencing of the 1.7 kb fragment was performed.

3.2 DETERMINATION OF THE DNA SEQUENCE OF *C. jejuni lex2B*

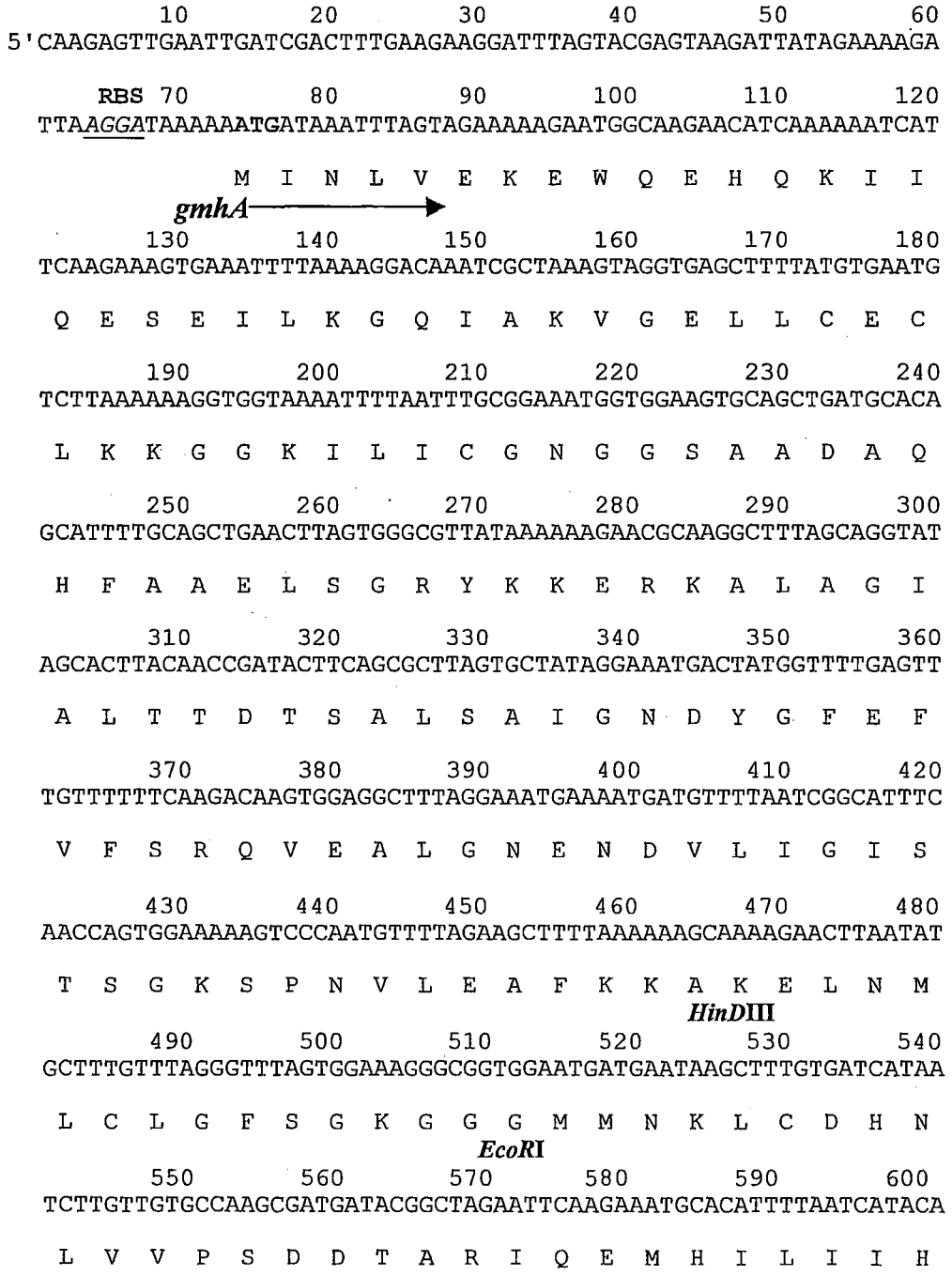
After proving that 1.7 kb fragment was capable of restoring WT LPS in a heptoseless *E. coli* (*gmhA*$^-$) strain the question was what made this fragment longer in *C. jejuni* 928

than in most other *C. jejuni* isolates. For that purpose DNA sequence analysis was conducted. The complete nucleotide sequence of 1.7 kb insert on pDJ4216 was determined for both DNA strands (primers T7 and 98.17; primers 98.21 and Sp6 (see section 2.10)). Nucleotide sequencing also oriented the gene order on the plasmid pDJ4216.

Analysis of DNA sequence revealed the presence of two complete ORFs (Figure 3.1). The first ORF is 561 nucleotides in length and is capable of encoding a polypeptide of 187 amino acids with a calculated molecular mass of 21 kDa (Figure 3.1). The ORF is terminated by a double stop codon (TAATGA). Six bases upstream of the proposed ATG initiation codon is a potential Shine-Dalgarno ribosomal binding site (AGGA). Nucleotide sequence of this ORF was analysed using the BLAST server and Clustal V to determine if, once translated, any amino acid similarities exist between this ORF in *C. jejuni* 928 and the amino acid sequence from *C. coli* M275 and *C. jejuni* NTCT11168. Global analysis (entire sequence) of the amino acids composing the first *C. jejuni* 928 ORF revealed 94% identity and 97% similarity with GmhA of *C. coli* M275. Strong identity was also found with GmhA (98%) from *C. jejuni* NCTC1168 (Figure 3.2). Based on the complementation profile and extensive amino acid conservation of GmhA between *C. jejuni* and *C. coli*, the first ORF of *C. jejuni* 928 was proposed to be *gmhA*. The 561 nucleotides composing the *gmhA* gene have a mol% (G+C) content of 35.1% which is consistent with that reported for other *C. jejuni* genes (Nuijten, 1990).

The second ORF is positioned downstream of the *gmhA* gene. It is 765 nucleotides in size and is transcribed in the same direction as *gmhA*. Inspection of the sequence upstream of the methionine initiation codon (within *gmhA*) or upstream of *gmhA* failed to reveal potential –35 and –10 consensus sequences. This would suggest the ORFs are transcriptionally coupled, perhaps as part of a larger operon, unlike the situation reported for the *gmhA* in *E. coli* which is unlinked to other genes (Brooke, 1996). The second ORF was capable of encoding a polypeptide of 255 amino acids, with a calculated molecular mass of 30.57 kDa. The ATG start codon overlaps the 3' end of *gmhA* gene by three bases (Figure 3.1). This ORF is terminated by an ochre termination

codon (TAA). Seven bases upstream of the proposed methionine initiation codon is a potential ribosomal binding site (GAGG). The 765 nucleotides composing this ORF have a mol% (G+C) of 25.5% which is slightly more AT-rich than that of the rest of the *C. jejuni* chromosome (Nuijten, 1990).



```
       610        620   RBS 630        640        650        660
CACACTTTGTCAGATCATCGATGAGGGTTTTTAATGAAAGTTTTTATCATAAATTTAGAG
                                  M  K  V  F  I  I  N  L  E
 T  L  C  Q  I  I  D  E  G  F  *  *
                                  lex2B

       670        680        690        700 HindIII 710        720
CGTTCTTTAGATAGGAAAGAGCATATGAAAAAACAAATTCAAAAGCTTTTTGAAAAAAAT
R  S  L  D  R  K  E  H  M  K  K  Q  I  Q  K  L  F  E  K  N

       730        740        750        760        770        780
CCTAGTTTAAAAAATAAATTAGAATTTATTTTCTTTAAAGCTATTGATGCTAAAAATAAA
P  S  L  K  N  K  L  E  F  I  F  F  K  A  I  D  A  K  N  K

       790        800        810        820        830        840
GAACATTTGGAATTTAAAGATCATTTTCCTTGGTGGGGTTCTTGGGTTTTGGGTAGAGAG
E  H  L  E  F  K  D  H  F  P  W  W  G  S  W  V  L  G  R  E
                  HindIII
       850        860        870        880        890        900
CTTTCTGATGGAGAGAAAGCTTGTTTTGCTAGTCATTATAAACTTTGGCAAGAATGTGTG
L  S  D  G  E  K  A  C  F  A  S  H  Y  K  L  W  Q  E  C  V

HindIII 910        920        930        940        950        960
AAGCTTGATGAGCCTATAATCATCCTAGAGGATGATGTGGAATTTAGTGATGAATTTTTA
K  L  D  E  P  I  I  I  L  E  D  D  V  E  F  S  D  E  F  L

       970        980        990       1000       1010       1020
AATAATGGAGTAGAATATATAGATGAGCTATTAAAGAGCAAATATGAATATATAAGACTT
N  N  G  V  E  Y  I  D  E  L  L  K  S  K  Y  E  Y  I  R  L

      1030       1040       1050       1060       1070       1080
TGTTATTTATTTGATAAAAGATTGTATTTTTTGAGCGAGAGTGGATATTATTTAAGTATT
C  Y  L  F  D  K  R  L  Y  F  L  S  E  S  G  Y  Y  L  S  I
                                                 PstI
      1090       1100       1110       1120       1130       1140
GAAAAACTTGCTGGAACTCAAGGCTATGTACTGCAAGTAAGTGCTGCAGTGAAATTTCTT
E  K  L  A  G  T  Q  G  Y  V  L  Q  V  S  A  A  V  K  F  L

      1150       1160       1170       1180       1190       1200
AAATATGCTAAAAATTGGATCTATGCTGTAGATGATTATATGGATATGTTTTATAAACAT
K  Y  A  K  N  W  I  Y  A  V  D  D  Y  M  D  M  F  Y  K  H

      1210       1220       1230       1240       1250       1260
AATGTGTTAAATATAGTTAAAAAGCCTTTATTTCTTAAGCAAGCAAATTTTTCAAGTGTT
N  V  L  N  I  V  K  K  P  L  F  L  K  Q  A  N  F  S  S  V

      1270       1280       1290       1300       1310       1320
ATTGTAGAATATGGAAGAAAATTTTCAATAAAATTAATATTATATAAAAAAATTGCTAGA
I  V  E  Y  G  R  K  F  S  I  K  L  I  L  Y  K  K  I  A  R

      1330       1340       1350       1360       1370       1380
GAAATTTTTAGATTTTATTCAAGTATTTTAAGGTTACTATTTATTATATATATAAAAAAT
E  I  F  R  F  Y  S  S  I  L  R  L  L  F  I  I  Y  I  K  N
```

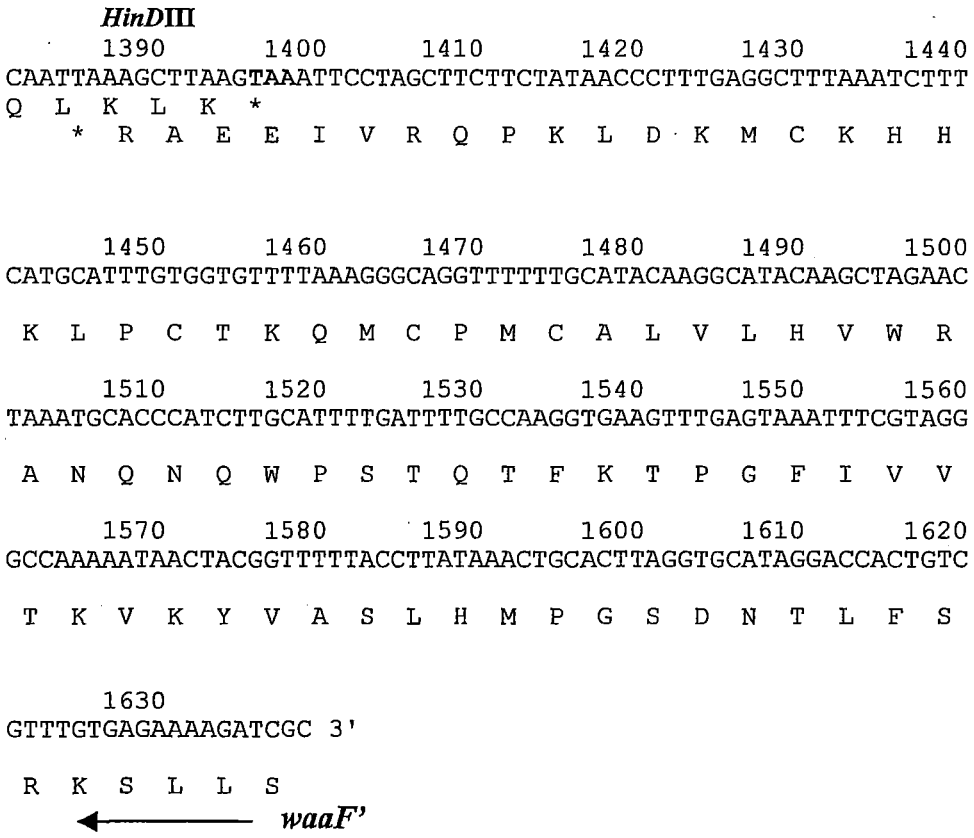


Figure 3.1. Nucleotide sequence and deduced amino acid sequence of *C. jejuni* 928 DNA spanning primers 96.01 to 94-293 (numbered from the 5' end). The translated nucleotide sequence shows two complete open reading frames (ORFs) that most likely are *gmhA* and *lex2B* and the partial ORF is *waaF* transcribed in an opposing direction. The bold, underlined sequence (AGGA) and (GAGG) denote possible ribosomal binding sites (RBS). Start and stop codons are indicated in bold for the respective ORFs. Restriction endonuclease sites *Pst*I, *Eco*RI *Hind* III are indicated in bold and labelled accordingly. The T7 promoter is at 5' end of the fragment while the Sp6 promoter is at 3' end.The deduced amino acid sequence reported in single letter code is defined: A (alanine); R (arginine); N (asparagine); D (aspartate); C (cysteine); Q (glutamine); E (glutamate); G (glycine); H (histidine); I (isoleucine); L (leucine); K (lysine); M (methionine); F (phenylalanine); P (proline); S (serine); T (threonine); W (tryptophan); Y (tyrosine); V (valine).

BLAST analysis (tblastx) of the second ORF contained within pDJ4216 revealed three regions in the translated polypeptide that exhibited approximately 50% identity and 70% similarity to the Lex2B protein from *H. influenzae*. The first region, encompassing amino acids 6 to 32, has 55% identity and 74% similarity to *H. influenzae* Lex2B. The second region, amino acids 67 to 109, revealed 53% identity and 76% similarity and the third region (amino acids 155 to193) displayed 46% identity and 56% similarity to the *H. influenzae* Lex2B (Figure 3.3, Appendix III). This data suggests that some domains

within the amino acid sequences between the two species are conserved. The putative *C. jejuni* 928 Lex2B also shows extensive conservation with glycosyl transferases such as the Lob1 from *H. somnus* (average 42% identity), Lex2B (*H. pylori*), LgtB (*N. gonorrhoeae*) and LgtE (*N. meningitidis*) (Figure 3.3, Appendix III). Interestingly *C. jejuni* 928 Lex2B shows only 72% identity and 80% similarity, at the amino acid level, to Lex2B from *C. coli* M275 (Figure 3.4 B). However at the nucleotide sequence level there is 84% identity (Figure 3.4 A).

Analysis of the remaining nucleotide sequence revealed a partial ORF (of 241 nucleotides) transcribed in an opposing direction, capable of encoding 84 amino acids (Figure 3.1). The ORF ends with an ochre (TAA) termination codon overlapping the stop codon of the *lex2B*. As expected, the presence of an initiation codon was not observed with the sequence primer 94-293 occuring within the coding region of the *waaF* gene *C. jejuni* NCTC11168. BLAST analysis of the partial ORF contained within pDJ4216 after the *lex2B* termination codon revealed a 40 amino acid segment that exhibited 31% identity and 54% similarity to WaaF proteins from *E. coli* and *S. typhimurium*. A 57 amino acid region showed 41% identity and 64% similarity to the WaaF protein of *H. pylori* (Appendix III).

Translation of deduced sequences for the two complete ORFs and one partial ORF suggest that the order of genes on the 1.7 kb *C. jejuni* 928 PCR amplicon as *gmhA-lex2B-waaF*. Clearly *C. jejuni* 928 contained a gene which had a high degree of identity to *lex2B* from numerous bacterial species. What is unclear, however, was whether this gene also existed in other *C. jejuni* strains, but in a heterologous location. Southern analysis was used to determine this (section 3.3).

```
A.
Cjej 928        ATGATAAATTTAGTAGAAAAAGAATGGCAAGAACATCAAAAAATCATTCA
Cjej1958        ATGATAAATTTAGTGGAAAAAGAATGGCAAGAACATCAAAAAATTGTTCA
Ccol M275       ATGATAAATTTAGTAGAAAAAGAATGGCAAGAACATCAAAAAGTTGCCGA
                ************** *************************** *     *

Cjej 928        AGAAAGTGAAATTTTAAAAGGACAAATCGCTAAAGTAGGTGAGCTTTTAT
Cjej1958        AGCAAGCGAAATTTTAAAAGGACAAATCGCTAAAGTAGGTGAGCTTTTGT
Ccol M275       GGAAAGTGAAATTTTAAAAGGGCAAATCGCAAAAGTAGGCGAGCTTTTGT
                 * *** ************** ******** ******** ******** *

Cjej 928        GTGAATGTCTTAAAAAAGGTGGTAAAATTTTAATTTGCGGAAATGGTGGA
Cjej1958        GTGAGTGTCTTAAAAAAGGTGGTAAAATTTTAATTTGCGGAAACGGTGGA
Ccol M275       ATGAGTGTTTAAAAAAAGGTGGAAAAATTTTAATTTGTGGAAATGGTGGG
                 *** *** * *********** ************** ***** *****

Cjej 928        AGTGCAGCTGATGCACAGCATTTTGCAGCTGAACTTAGTGGGCGTTATAA
Cjej1958        AGTGCGGCTGATGCTCAGCATTTTGCGGCTGAACTTAGCGGGCGTTATAA
Ccol M275       AGTGCCGCGGATTCTCAACATTTTGCAGCGGAGCTTAGCGGCAGATATAA
                ***** ** *** * ** ******** ** ** ***** **  * *****

Cjej 928        AAAAGAACGCAAGGCTTTAGCAGGTATAGCACTTACAACCGATACTTCAG
Cjej1958        AAAAGAACGCAAAGCTTTAGCAGGCATAGCACTTACAACCGATACTTCAG
Ccol M275       AAAAGAACGCAAAGCTTTAGCGGGTATAGCTTTGACAACAGACACTTCAC
                ************ ******** ** *****  * ***** ** ******

Cjej 928        CGCTTAGTGCTATAGGAAATGACTATGGTTTTGAGTTTGTTTTTTCAAGA
Cjej1958        CACTTAGTGCCATAGGAAATGACTATGGTTTTGAGTTTGTTTTTTCAAGA
Ccol M275       GACTTAGTGCTATAGGCAATGATTATGGATTTGAGTTTGTTTTTTCAAGG
                  ******** ***** ***** ***** ********************

Cjej 928        CAAGTGGAGGCTTTAGGAAATGAAAATGATGTTTTAATCGGCATTTCAAC
Cjej1958        CAAGTGGAAGCTTTAGGGAATGAAAAGGATGTTTTAATCGGTATTTCAAC
Ccol M275       CAAGTAGAAGCTTTGGGAAGTGAAAATGATATTTTAATAGGCATTTCAAC
                ***** ** ***** ** * ****** *** ******* ** ********

Cjej 928        CAGTGGAAAAAGTCCCAATGTTTTAGAAGCTTTTAAAAAAGCAAAAGAAC
Cjej1958        GAGCGGAAAAAGCCCTAATGTTTTAGAAGCTTTAAAAAAAGCAAAAGAGC
Ccol M275       CAGTGGAAAAAGTCCTAATGTCTTAGAAGCATTTAAAAAAGCTAAAGAAT
                 ** ******** ** ***** ******** ** ******** *****

Cjej 928        TTAATATGCTTTGTTTAGGGTTTAGTGGAAAGGGCGGTGGAATGATGAAT
Cjej1958        TTAATATGCTTTGTCTAGGGCTTAGCGGAAAAGGTGGTGGAATGATGAAT
Ccol M275       TAAATATGCTTTGCTTGGGGCTTAGTGGCAAAGGAGGTGGAAAGATGAAT
                * ***********  * *** **** ** ** ** ******* *******

Cjej 928        AAGCTTTGTGATCATAATCTTGTTGTGCCAAGCGATGATACGGCTAGAAT
Cjej1958        AAGCTTTGTGATCATAATCTTGTTGTGCCAAGCGATGATACAGCTAGAAT
Ccol M275       GAACTTTGCGATCATAATCTTGTTGTGCCAAGCGATGATACGGCTAGAAT
                 * ***** ********************************* ********

Cjej 928        TCAAGAAATGCACATTTTAATCATACACACACTTTGTCAGATCATCGATG
Cjej1958        TCAAGAAATGCACATTTTAATCATACACACACTTTGTCAGATCATAGATG
Ccol M275       TCAAGAAATGCATATCTTAATCATACACACGCTTTGTCAGATAGTAGATG
                ************ ** ************** ***********  * ****

Cjej 928        AGGGTTTTT-A
Cjej1958        AGAGTTTTT-A
Ccol M275       AGAGCTTTTGA
                ** * **** *
```

B.

```
Cjej928      1 MINLVEKEWQEHQKIIQESEILKGQIAKVGELLCECLKKGGKILICGNGGSAADAQHFAA
CcolM275     1 MINLVEKEWQEHQKVAEESEILKGQIAKVGELLYECLKKGGKILICGNGGSAADSQHFAA
Cjej1958     1 MINLVEKEWQEHQKIVQASEILKGQIAKVGELLCECLKKGGKILICGNGGSAADAQHFAA

Cjej928     61 ELSGRYKKERKALAGIALTTDTSALSAIGNDYGFEFVFSRQVEALGNENDVLIGISTSGK
CcolM275    61 ELSGRYKKERKALAGIALTTDTSALSAIGNDYGFEFVFSRQVEALGSENDILIGISTSGK
Cjej1958    61 ELSGRYKKERKALAGIALTTDTSALSAIGNDYGFEFVFSRQVEALGNEKDVLIGISTSGK

Cjej928    121 SPNVLEAFKKAKELNMLCLGFSGKGGGMMNKLCDHNLVVPSDDTARIQEMHILIIHTLCQ
CcolM275   121 SPNVLEAFKKAKELNMLCLGLSGKGGGKMNELCDHNLVVPSDDTARIQEMHILIIHTLCQ
Cjej1958   121 SPNVLEALKKAKELNMLCLGLSGKGGGMMNKLCDHNLVVPSDDTARIQEMHILIIHTLCQ

Cjej928    181 IIDEGF
CcolM275   181 IVDESF
Cjej1958   181 IIDESF
```

Figure 3.2. Alignment of *C. jejuni* 928 (Cjej928), *C. coli* M275 (CcolM275) and *C. jejuni* NCTC1168 (Cjej1958) nucleotide (*gmhA*) and amino acid (GmhA) sequences).
Panel A. Nucleotide sequence alignment.
Panel B. Amino acid sequence alignment
Gaps, indicated by dashes, were introduced to obtain maximal alignment; "*" indicates identical nucleotides;

- indicates identical amino acids
- indicates related amino acids
- indicates different amino acids

```
lex2B928     1 M-------------------------------------KVFIINLERSLDR--KEHMK
lex2BHi      1 MFIT----------------------------------PIFIINLEKSTDR--KAYMQ
lob1Hs       1 MNQSINQSINQSINQSINQSINQSINQSINQSINQSVIFVINLEKATER--KHFIS
lex2BHp      1 M-RVFAISLNQKVCDTFGLVFRDTT--TLLNSINATHHQAQIFDAIYSKTFEGGLHPLVK
lgtBNg       1 M-----------------------------------QNHV--ISLASAAER------R
lgtENm       1 M-----------------------------------QNHV--ISLASAAER------R

lex2B928    20 KQIQKLFEKNPSLKNKLEFIFFKAIDAKNKEHLEFKDHFPWWGSWVL-GRE-LSDGEKAC
lex2BHi     23 AQFELLFSNN--LI--QEIHFFDAIYGKSNPNHPLFQRYNENKRLNAKGYP-LTLGQLGC
lob1Hs      59 HQFTALQEQHPDIV--INYQFFTGVNGNTQPNHPLFAKYNQKKRYQRKGNE-ITLGQLGC
lex2BHp     58 KHLHPYFITQNIKDMGITTNLISEVS-------KFYYALKYHAKF-------MSLGELGC
lgtBNg      16 AHIAATFGSR-----GIPFQFFDALMPSERLERAMAELVP-----GLSAHPYLSGVEKAC
lgtENm      16 AHIADTFGAH-----DIPFQFFDALMPSEALEQAMAELVP-----GLSAHPYLSGVEKAC

lex2B928    78 FASHYKLWQECVKLDEP-IIILEDDVEF---SDEFLNNGVEYIDELLKSKYEYIRLCYL-
lex2BHi     78 YASHYSMWEKCVELDYP-IIVLEDDAKF---KNNFLE-VLDFINSD-KNTFEFFWLL-PD
lob1Hs     116 YASHYLLWEKCVQLQQP-IIVLEDDAIL---QPNFLA-VYQFCFSA-ENQFQFFWLTHSN
lex2BHp    104 YASHYSLWEKCIELNEA-ICILEDDITL---KEDF-KEGLDFLEKHIQE-LGYIRLMHLL
lgtBNg      66 FMSHAVLWEQALDEGVPYIAVFEDDVLLGEGAEQFLAED-TWLQERFDPDSAFV----VR
lgtENm      66 FMSHAVLWKQALDEGLPYVAVFEDDVLLGKDAEKFLAED-TWLEERFDKDSAFI----VR

lex2B928   133 ---FDKRLYFLSESGYYLSIEKL----A---GTQGYVLQVSAAVKFLKY----AKNWIYA
lex2BHi    131 RLKNKRKLISNFGN---LSIYQFSKGFA---GATGYYLTPQAARKFLTQ----SKEWYLT
lob1Hs     170 SSKIRTKLIHTLPDSTKLEQHYF--GYS---NTTGYYLTPQAAQKFLDS----SQEWIYN
lex2BHp    158 YDASVKSEPLSHKNHEIQERVGIIKAYSEGVGTQGYVITPKIAKVFLKC----SRKWVVP
lgtBNg     121 LETMFMHVLTSPSGVADYGGRAFPLLESEHCGTAGYIISRKAMRFFLDRFAVLPPERLHP
lgtENm     121 LETMFAKVIVRPDKVLNYENRSFPLLESEHWGTAGYIISREAMRFFLERFAVLPAERIKA

lex2B928   179 VDDYM-DMFYKHNVLNI--VKKPLFLKQANFSS-------------VIVEYGRKFSIKLI
lex2BHi    181 VDVTM-DRFFENKVPPY--AIVPFCLED-DGEI-------------ESTIYEKQKKQR-S
lob1Hs     221 VDIFM-DRFYENHVALL--GVNPPCVKP-DFSK-------------QSQI-TMNKNNR-T
lex2BHp    214 VDTIM-DATFIHGVKNL--VLQPFVIADDEQIS-------------TIA----RKEEPYS
lgtBNg     181 VDLMMFGNPDDREGMPVCQLNPALCAQELHYAKFHDQNSALGSLIEHDRRLNRKQQWRDS
lgtENm     181 VDWMMFTYFFDKEGMPVYQVNPALCTQELHYAKFLSKNSMLGSDLEKDR----EQERRHR

lex2B928   223 LYKKIAREIFRFYSSILR------------LLFIIYIKNQLKLK
lex2BHi    223 LKIVIMRELFNLKTNIKR------------RIYNL-------FH
lob1Hs     262 FWVKLRREYFALLERIKR------------FVYWTC------YC
lex2BHp    254 PKIALMREL--------H------------FKYLKY----WQFV
lgtBNg     241 PANTFKHRLIRALTKIGREREKR--RQRREQL---IGKIIVPFQ
lgtENm     237 RSLKVMFDLKRALGKFGREKKKRMERQRQAELEKAYGRRVISFK
```

Figure 3.3. Amino acid sequence alignment of Lex2B homologous proteins from different bacterial sources: C. *jejuni* 928 (lex2B928); *H. influenzae* type b (lex2BHi) accession no. U05670 ; *H. somnus* (lob1Hs) accession no. U94833; *H. pylori* (lex2BHp) accession no. AE000594; *N. gonorrhoeae* (lgtBNg) accession no. U13554 and *N. meningitidis* (lgtENm) accession no. U25839. Amino acid sequences identified with BLAST by similarity to the deduced amino acid sequence from the *C. jejuni lex2B* homolog, were aligned (entire length) using Clustal V and displayed using Boxshade. Gaps, indicated by dashes, were introduced to obtain maximal alignment.

■ indicates identical amino acids

■ indicates related amino acids

■ indicates different amino acids

A.

```
Cjej928         ATGAAAGTTTTTATCATAAATTTAGAGCGTTCTTTAGATAGGAAAGAGCA
CcolM275        ATGAAGGCATTTATCATTAATTTAGAGCGTTCTTTAGATAGAAAAGAGTA
                ***** *  ******** ************************ ****** *

Cjej928         TATGAAAAAACAAATTCAAAAGCTTTTTGAAAAAAATCCTAGTTTAAAAA
CcolM275        CATGCAAAGGCAAAATCAAAAACTTTTTGAAAAGAACCCTAGTCTAAAAA
                 *** ***  **** ****** ********** ** ****** ******

Cjej928         ATAAATTAGAATTTATTTTCTTTAAAGCTATTGATGCTAAAAATAAAGAA
CcolM275        ATAAGCTAGAATTTATTTTCTTTAAAGCCGTTGATGCTAAAAATAAAGAA
                ****  ********************  ********************

Cjej928         CATTTGGAATTTAAAGATCATTTTCCTTGGTGGGGTTCTTGGGTTTTGGG
CcolM275        TATTTAGAATTTAAACAACATTTTCCTTGGTGGGCTTCTTGGGTTTTAGG
                 **** *********  * ***************** ************ **

Cjej928         TAGAGAGCTTTCTGATGGAGAGAAAGCTTGTTTTGCTAGTCATTATAAAC
CcolM275        TAGAGAGCTTAGTGATGGAGAAAAGGCTTGTTTTGCGAGTCATTATAAAC
                **********  ********* ** ********** **************

Cjej928         TTTGGCAAGAATGTGTGAAGCTTGATGAGCCTATAATCATCCTAGAGGAT
CcolM275        TTTGGCAAGAATGTATAAAACTTGATAAACCTATAATCATCCTAGAAGAT
                ************** * ** ****** * ***************** ***

Cjej928         GATGTGGAATTTAGTGATGAATTTTTAAATAATGGAGTAGAATATATAGA
CcolM275        GATGTTGAATTTAGTGATGAATTTTTAAATAATGGGGAAGAATACATAGA
                ***** ***************************** * ****** *****

Cjej928         TGAGCTATTAAAGAGCAAATATGAATATATAAGACTTTGTTATTTATTTG
CcolM275        AGAATTATCAAAGAGTGAGTATGAATATGTAAGATTTTGTTATTTGTTTG
                 **  *** ******  * ********* ***** ********** ****

Cjej928         ATAAAAGATTGTATTTTTTGAGCGAGAGTGGATATTATTTAAGTATTGAA
CcolM275        ATAAGAAATTTTATCCTTTAAATGAAAATT-ATATT--CTAAGTTTTGAA
                **** * *** ***  *** *  ** * *  *****   ***** *****

Cjej928         AAACTTGCTGGAACTCAAGGCTATGTACTGCAAGTAAGTGCTGCAGTGAA
CcolM275        AAACTTGCAGGAACTCAAGGTTACGTATTAAAACCTAGTGCTGCAAACAA
                ******** ********** ** *** *  **   *********   **

Cjej928         ATTTCTTAAATATGCTAAAAATTGGATCTATGCT-GTAGATGATTATATG
CcolM275        ATTTATATCAAAGGC-AAAATTTATTTATACACCCGTGGATGATTATATG
                **** *   * * ** **** **   * **  *  ** ************

Cjej928         GATATGTTTTATAAACATAATGTGTTAAATATAGTTAAAAAGCCTTTATT
CcolM275        GATATGTTTTATAAACATAAGGTATTAAATATCGTAAAAAAACCTTTATT
                ******************** ** ******** ** ***** ********

Cjej928         TCTTAAGCA-AGCA--AATTTTTCAAGTGTTATTGTAGAATATGGAAGAA
CcolM275        ATTAAAACATAATTGTAAATTAGAAAGCGAAATTTCTAATCTTGGACGTA
                  * ** ** *     ** **   *** *  ***    *   **** * *

Cjej928         AATTTTCAATAAAATTAATATTATATAAAAAAATTGCTAGAGAAATTTTT
CcolM275        CAAAT--AAAAAACTTAAAATT-CATAGAAAAATTGTAAGAGAATTTTTT
                 *  *  ** *** **** ***  *** ********  ****** *****

Cjej928         AGATTTTATTCAAGTATTTTAAGGTTACTATTTATTATATATATAAAA-A
CcolM275        AGACTTTATAGAGATATTTCAAGATTGCTCTATGTTTTTTATATTAAGTA
                *** *****  *  ***** *** ** ** * * ** * ***** **  *

Cjej928         ATCAATTAAAGCTTAAGTAA
CcolM275        AAAAATTAAATCTTAA----
                *  ******* *****
```

B.

```
Cjej928      1 MKVFIINLERSLDRKEHMKKQIQKLFEKNPSLKNKLEFIFFKAIDAKNKEHLEFKDHFPW
CcolM275     1 MKAFIINLERSLDRKEYMQRQNQKLFEKNPSLKNKLEFIFFKAVDAKNKEYLEFKQHFPW

Cjej928      61 WGSWVLGRELSDGEKACFASHYKLWQECVKLDEPIIILEDDVEFSDEFLNNGVEYIDELL
CcolM275     61 WASWVLGRELSDGEKACFASHYKLWQECIKLDKPIIILEDDVEFSDEFLNNGEEYIEELS

Cjej928     121 KSKYEYIRLCYLFDKRLYFLSESGYYLSIEKLAGTQGYVLQVSAAVKFLKYAKNWIYA-V
CcolM275    121 KSEYEYVRFCYLFDKKFYPLNEN-YILSFEKLAGTQGYVLKPSAANKFISKAK-FIYTPV

Cjej928     180 DDYMDMFYKHNVLNIVKKPLFLKQA-NFSSVIVEYGRKFSIKLILYKKIAREIFRFYSSI
CcolM275    179 DDYMDMFYKHKVLNIVKKPLLLKHNCKLESEISNLGRT-NKKLKIHRKIVREFFRLYRDI

Cjej928     239 LRLLFIIYIKNQLKLK
CcolM275    238 SRLLYVFYIK------
```

Figure 3.4. Alignment of *C. jejuni* 928 (Cjej928) and *C. coli* M275 (CcolM275) nucleotide (*lex2B*) and amino acids (Lex2B) sequence.
Panel A. Nucleotide sequence alignment.
Panel B. Amino acid sequence alignment
Gaps, indicated by dashes, were introduced to obtain maximal alignment and may also indicate regions of unobtained sequence. "*" indicates identical nucleotides;

- indicates identical amino acids
- indicates related amino acids
- indicates different amino acids

3.2.1 HYDROPHOBIC CLUSTER ANALYSIS (HCA)

Hydrophobic cluster analysis (HCA) was chosen as a method to search for secondary structural motifs within the Lex2B protein of *C. jejuni* 928 and for comparison of this protein with some well established glycosyl transferases such as LgtB from *N. gonorrhoeae*. This method relies upon a two-dimensional (2D) representation of protein sequence (Gaboriaud, 1987). HCA is not based primarily on the maximization of the amino acid identity, but rather on the detection and comparison of the structural segments constituting the hydrophobic core of globular protein domains, with or without trans-membrane regions (Lemesle-Varloot, 1990). Between hydrophobic clusters are loops, which are characterized by a high content of strong hydrophilic amino acids (DENQHKR) as well as in proline, glycine, serine and threonine.

A Blast search of the deduced *C. jejuni* 928 Lex2B protein sequence identified similarity to a variety of known and putative β-glycosyl transferases (Figure 3.3). Recently Heinrichs *et al.* (Heinrichs, 1998), characterised the WaaX protein from *E. coli* R4 as a galactosyl transferase involved in the attachment of a side branch β-linked Gal to Glc II in outer domain of the core OS. Blast analysis did not reveal any similarity between this protein and Lex2B from *C. jejuni* 928. However, this protein was included in the HCA analyses since WaaX showed 2D similarity with LgtB and LgtE galactosyl transferases from *N. gonorrhoeae* and *N. meningitidis*. These two galactosyl transferases showed ~ 35% identity in 1D alignments with the Lex2B protein from *C. jejuni* 928. Results obtained from HCA plots (Figure 3.5) highlighted similarity between Lex2B and LgtB and LgtE, but also with the unrelated protein WaaX. Similarity is evident within the N-terminal 110 amino acids. In particular, two conserved Arg residues in the N-terminus, a Phe-X-Phe-Phe motif 30-40 amino acids from N-terminus, and a Glu-Asp-Asp motif located 90-110 amino acids from N-terminus, were identified as consensus sequence features of the galactosyl transferase family. A representive of this family, LgtB, catalyzes the formation of β-D-Gal-(1-4)-α-D-GlcNAc linkage in the the synthesis of LOS in *N. meningitidis*. HCA plots of Lex2B, LgtB and WaaX indicate that the conserved sequence features of the proteins exist in similar structural regions, particulary in their potential catalytic residues presented with Asp^{30-40}, $Glu\text{-}Asp\text{-}Asp^{90-110}$ and their occurrence in flexible loop structures.

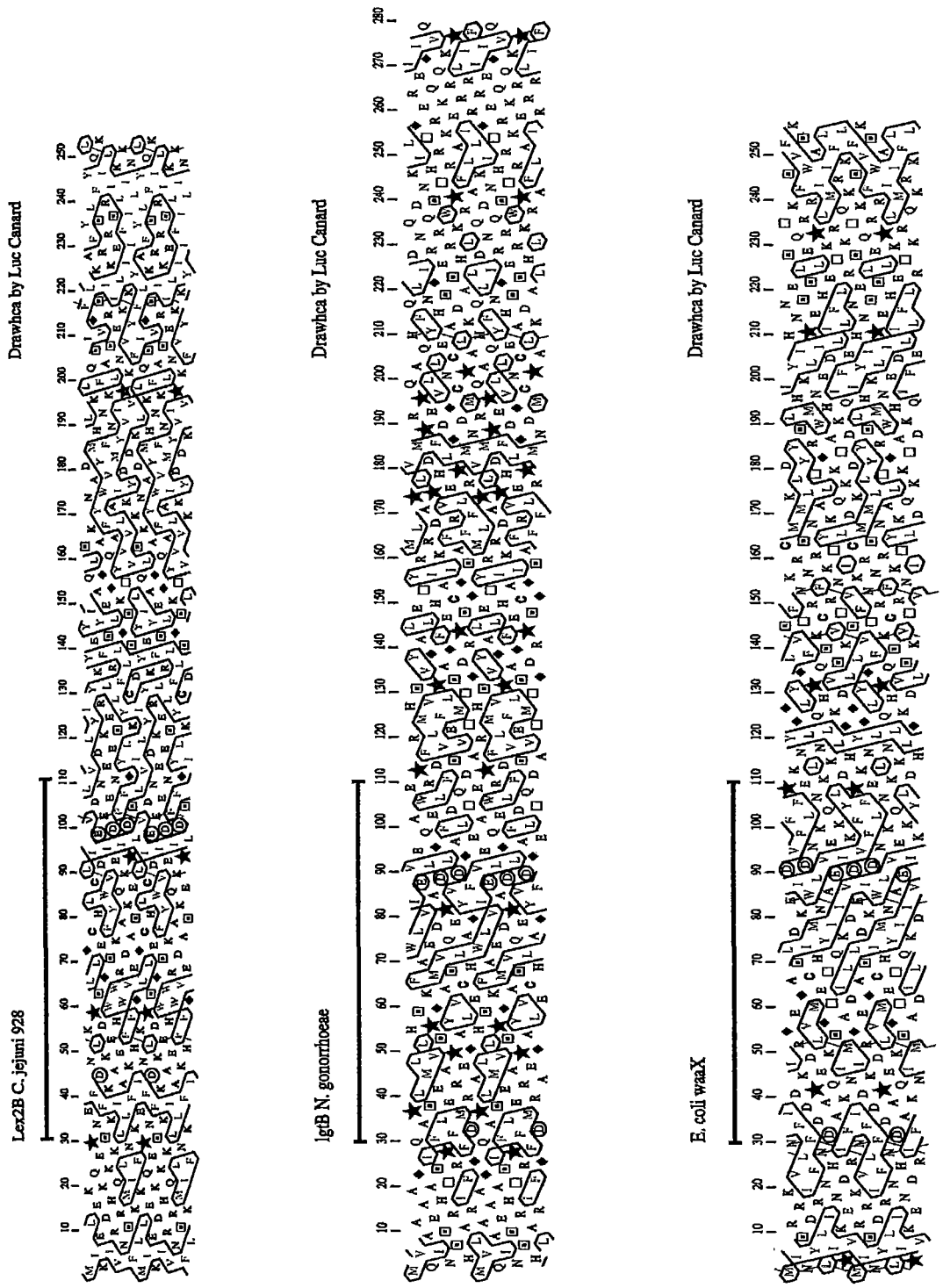


Figure 3.5. Alignments of HCA plots of Lex2B, LgtB and WaaX. Plots were made using the HCA-Plot program (Doriane Informatique, Le Chesnay, France). The protein sequence is written on a duplicated α-helical net, and clusters of hydrophobic amino acids are circled. A comprehensive description of the application of the program can be found in recent review (Callebaut, 1997). Association of colours and signs with amino acids are described in Table 3.1.

Table 3.1. Classification of amino acids for HCA* (one letter code)

Hydrophobic	Strong: VILF (green); Medium: WMY (green); Mimetic: AC (black)
Hydrophilic	DENQ (red); HKR (blue)
Special	P (¬)(red); G (♦) (black); T ( ) (black); S (♣) (black)

*For the programs that produce color plots, the color codes are indicated in parentheses

3.3 SOUTHERN HYBRIDISATION ANALYSIS

Southern hybridisation of genomic DNA from *C. jejuni* strain 928 and *C. jejuni* NCTC11168 were performed to confirm that DNA rearrangement had not occurred during the constructionof pDJ4216, to determine the copy number of *lex2B* on the *C. jejuni* 928 chromosome and also to confirm the absence of *lex2B* on the NCTC11168 chromosome. A 0.477 kb PCR amplicon generated from *C. jejuni* 928 DNA using the primers 98.21 and 94.375 (see Table 2.5) was used to construct an internal probe to *lex2B* (Figure 3.6). To confirm that transfer of *C. jejuni* NCTC11168 DNA had occurred, the membrane was stripped and rehybridised with an internal probe for the 16S rDNA gene. The second probe was a ~0.5 kb PCR amplicon generated from *C. jejuni* NCTC11168 DNA using the primers 97.21 and 97.23 (see Table 2.5). *C. jejuni* 928 and *C. jejuni* NCTC11168 chromosomal DNA were restriction endonuclease digested using *Pst* I, *HinD* III and *Nde* I for *C. jejuni* 928 and *Pst* I for *C. jejuni* NCTC11168. Fragments were resolved by agarose gel electrophoresis (see section 2.9).

The *lex2B* internal probe was observed to hybridise to a *Pst* I fragment of approximately 4.8 kb and to *HinD* III fragments of ~0.46 kb, 0.18 kb and 0.15 kb (Figure 3.7). From nucleotide sequence data one more *HinD* III fragment (42 bp in size) should have hybridised with the probe. A band of this size has not been observed to hybridise with the probe and there is a strong probability that the fragment did not transfer onto the membrane due to small size of this fragment. The Southern profile is consistent with the physical map of *C. jejuni* 928 *lex2B* (Figure 3.5). Hybridisation of the *lex2B* probe to *C. jejuni* NCTC11168 DNA fragments was not observed. This result is consistent with the reported absence of *lex2B* on the *C. jejuni* NCTC11168 chromosome (Upritchard, 1997;

Sanger, 1998). Hybridisation to a single large *Pst* I fragment at least as large as the 1.1 kb was predicted, since one *Pst* I site exists (within *lex2B*) on the entire insert in pDJ4216 (Figure 3.6) and the probe binds upstream of the *Pst* I site. Frequent occurrence of *HinD* III fragments (restriction sequence AAGCTT) is due to low mol% (G+C) of *lex2B*. Restriction endonuclease *HinD* III was chosen because other restriction enzymes (e.g. *Nde* I) do not digest *C. jejuni* 928 DNA into small fragments. One probable reason for incomplete digestion by *Nde I* is that this restriction enzyme requires very pure DNA.

Hybridisation with the 16S rDNA probe showed expected results. Hybridisation of the probe to both *C. jejuni* strains DNA resulted in detection of several bands. Multiple hybridised fragments are expected because the 16S rDNA is present in several copies on the *C. jejuni* chromosome.

Collectively, Southern hybridisation indicates *lex2B* is present on the *C. jejuni* 928 chromosome as a single copy and rearrangements of nucleotide sequence had not occurred during the cloning procedure. Absence of hybridisation to the *C. jejuni* NCTC11168 genomic DNA with a *lex2B* probe strongly suggests that *lex2B* does not exist within this strain.

Although strain 928 contained what appeared to be a novel gene (relative to the sequenced strain), it remained unclear whether this gene was functional or a non-transcribed artifact. RT-PCR of extracted RNA, using primers specific for *lex2B*, was utilised to answer this question.

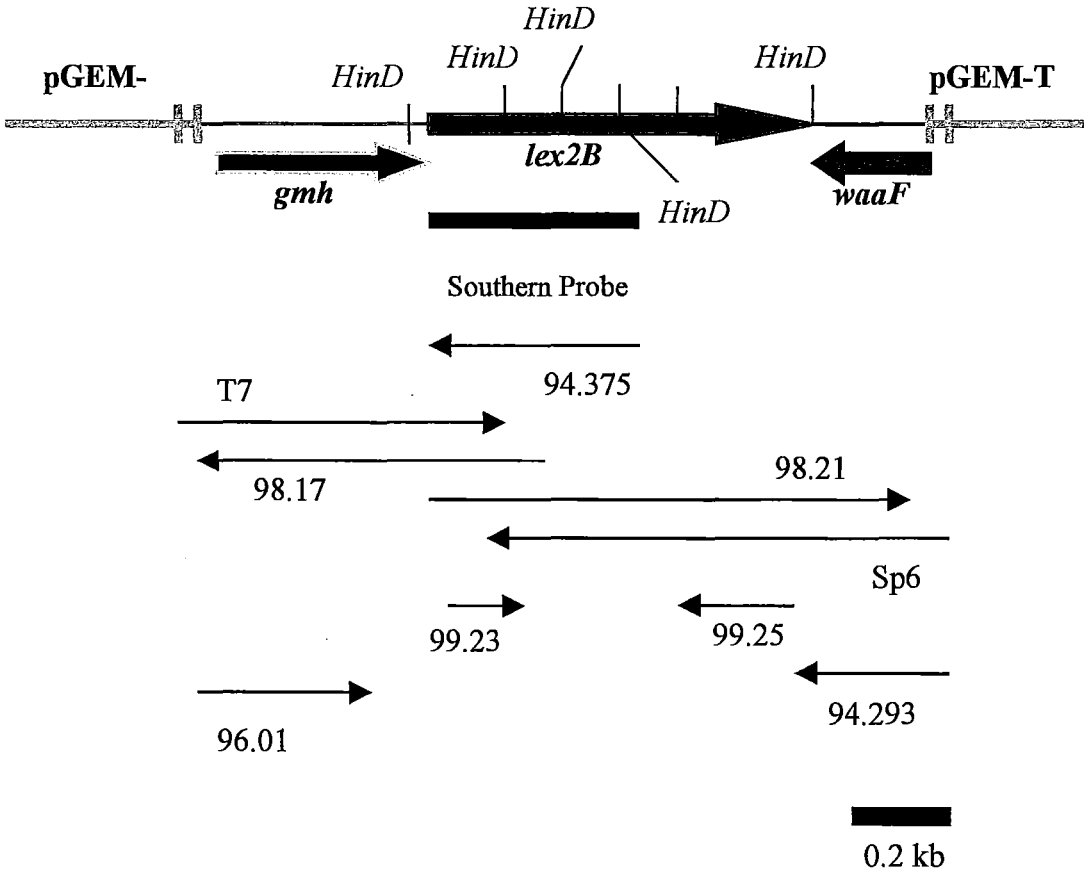


Figure 3.6. Physical map showing the PCR amplicon generated from *C. jejuni* 928 using single stranded oligodeoxynucleotide primers 96.01 and 94.293. Purple solid lanes indicate the ends of the pGEM-T vector sequence. Grey solid lines indicate the insert sequence. The physical map shows gene designations, coding regions and direction of transcription (arrows) and relevant restriction sites. Black arrows beneath the restriction map indicate nucleotide sequence generated from pDJ4216 with primers T7, Sp6, 98.21, 98.17, 94.375, 99.23 and 99.25. The size and annealing position in pDJ4216 of the *lex2B* southern probe (constructed using primers 98-21/94-375) is indicated. Map size is indicated by a scale bar, in kilobases, to the right.

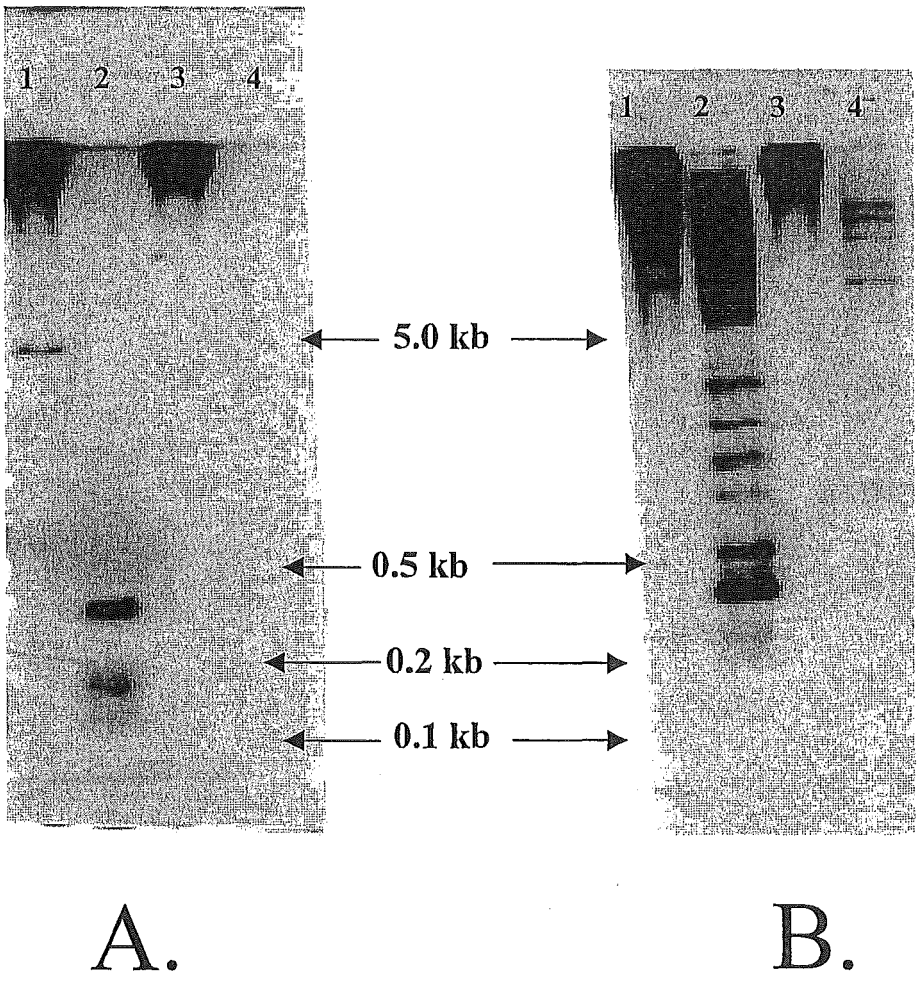


Figure 3.7. Southern hybridisation analysis of genomic DNA from *C. jejuni* strains 928 and NCTC11168. The DNA was restriction digested with *Pst*I (lanes 1 and 4), *HinD*III (lane 2) and *Nde*I (lane 3), separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with a 0.48 kb DIG labelled PCR amplicon internal to the *lex2B* gene (**panel A**). Afterwards, the membrane was stripped and reprobed with 0.5 kb DIG labelled PCR amplicon internal for 16S rRNA gene (**panel B**). Lanes: 1-3) *C. jejuni* 928; 4) *C. jejuni* NCTC11168.

3.4 DETECTION OF *lex2B* mRNA BY RT-PCR

Detection of mRNA by reverse transcription-PCR (RT-PCR) was used to monitor *lex2B* gene expression. The method combines two sequential enzymatic steps: the syntheses of DNA (cDNA) from the RNA template by reverse transcriptase (RT) followed by conventional PCR using a heat-stable DNA polymerase (*Taq* polymerase). The extremely high sensitivity of RT-PCR enables detection of rare mRNA, mRNA produced in small quantities or in small numbers of cells (Siebert, 1993).

Total RNA was isolated from *C. jejuni* 928 and *C. jejuni* NCTC11168 (see section 2.7) and digested with *DNase*I. Untreated samples and *DNase*I treated total RNA were subjected to an electrical current through 1% agarose gel, and subsequently stained with ethidium bromide (see section 2.4.4) to visualise (Figure 3.8). Results show that while untreated RNA is contaminated with chromosomal DNA, *DNase*I treated RNA removes this contamination.

To investigate specific *lex2B* mRNA production, RT-PCR was performed on total RNA, isolated from either *C. jejuni* strains 928 and NCTC11168, treated with *DNase*I. Because even minuscule amounts of contaminating DNA (<1%) can produce a false-positive amplification signal in RT-PCR, total RNA was, prior to synthesis of cDNA, aliquoted in three tubes and treated with either *RNase*A, *DNase*I or *DNase*I/*RNase*A. These enzyme reaction mixtures included all reagents for cDNA synthesis (see section 2.7) except reverse transcriptase to avoid contamination with possible DNA in these reagents. After synthesis of cDNA, PCR was performed with internal primers for the *lex2B* RNA (98-21 and 94-375) (see Table 2.5). PCR amplification of chromosomal *C. jejuni* 928 DNA was also included as a positive control, along with a control for PCR reagents and primers (Figure 3.9). To confirm that absence of the amplification signal in *DNase*I treated RNA samples from *C. jejuni* NCTC11168 was not due to an absence of total RNA, PCR was carried out with primers for 16S rDNA (97.21and 97.23- see Table 2.5) (Figure 3.10).

Figure 3.9 shows the results of the RT-PCR with *lex2B* internal primers. Amplification of a 480 bp fragment (lane 2) was consistent with the expectation that *lex2B* was transcribed in *C. jejuni* 928. The size of the fragment and restriction endonuclease digestion profile generated with *StyI* (data not shown) were consistent with the predicted size of a PCR fragment estimated from the physical map of *lex2B* from *C. jejuni* 928. The absence of a signal from samples treated with *RNase*A (lane 3) and *DNase*I/*Rnase*A (lane 4) verified that amplification of the sample treated only with *DNase*I is due to mRNA from *lex2B*. The lack of a PCR amplicon from *C. jejuni* NCTC11168 RNA with *lex2B* internal primers (lane 5) was predicted because NCTC11168 had not shown presence of *lex2B* on its chromosome (by PCR and Southern).

A control experiment with 16S rDNA primers strongly suggests that the *C. jejuni* NCTC11168 lack of amplification with *lex2B* primers was not due to a lack of RNA but absence of *lex2B* mRNA in NCTC11168 (Figure 3.10).

To investigate the speculation that the *gmhA* and the *lex2B* are transcribed for the same promoter, RT-PCR was conducted on *C. jejuni* 928 RNA treated with *DNase*I using primers 97.01 (within *gmhA*), *gmhD*DV (*gmhD*) and 94.375 (within *lex2B*) (see Table 2.5). Preliminary results suggest that *lex2B* is not transcriptionaly coupled with upstream genes (data not shown). However these results may be incorrect since expected amplicon of 3.0 kb, if genes are transcribed from the same promoter, is hard to obtain due to short half-life of mRNA. Also with PCR conditions used in this study it is unlikely a long fragment would be generated. Unfortunately time constraints prevented futher characterisation of the promoter of *lex2B*.

In general, RT-PCR had shown that *lex2B* in *C. jejuni* 928 is likely to be functional and confirmed previous results (DNA sequencing and Southern hybridisation) that information for Lex2B synthesis does not exist in *C. jejuni* NCTC11168. Investigation of a transcriptional connection of *lex2B* with upsteam genes (*gmhA*, *gmhB/C* and *gmhD*) suggests that *lex2B* is not transcribed from the same promoter as these genes, although more work must be performed because to obtain large PCR amplicons (which would be the case if *lex2B* is transcribed with *gmhA* and upstream genes) alternate PCR protocols should be trialled.

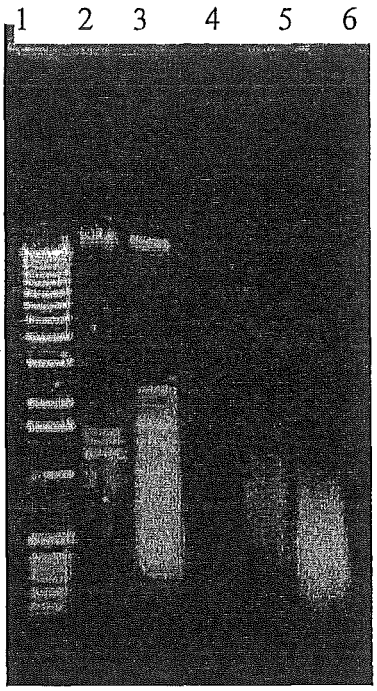


Figure 3.8. Total RNA from *C. jejuni* isolates 928 and NCTC11168. Lanes: **1)** 1 kb ladder; **2)** NCTC11168 RNA; **3)** 928 RNA; **4)** blank; **5)** NCTC11168 *DNase*I treated RNA; **6)** 928 *DNase*I treated RNA. Samples were subjected to an electrical current through 0.8% agarose gel and visualised using ethidium bromide staining and UV light (see section 2.4.4).

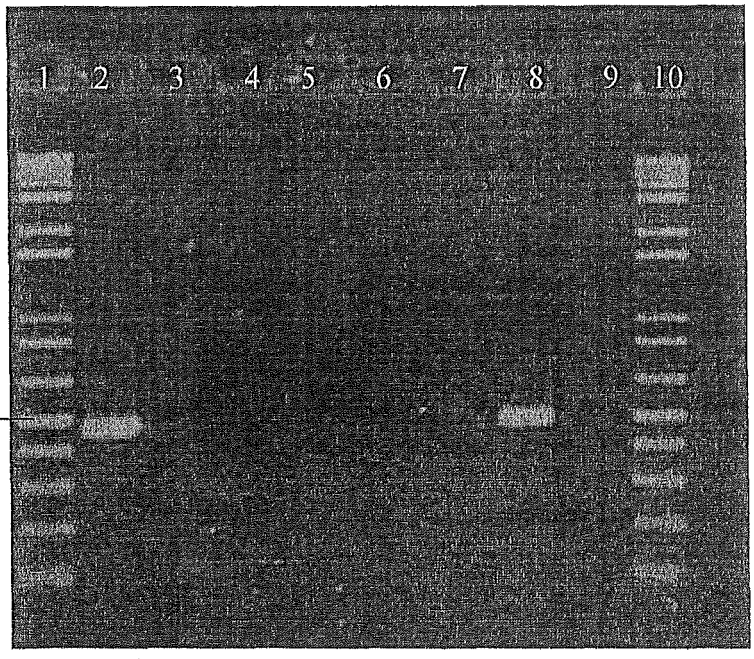


Figure 3.9. RT-PCR of *C. jejuni* 928 mRNA and *C. jejuni* NCTC11168 mRNA with *lex2B* internal primers. Lanes: 1) and 10) 1 kb plus ladder; 2) 928 RNA treated with *DNAse*I; 3) 928 RNA treated with *RNase*A; 4) 928 RNA treated with *DNase*I/*RNase*A; 5) NCTC11168 RNA treated with *DNase*I; 6) NCTC11168 RNA treated with *RNase*A; 7) NCTC11168 RNA treated with *DNase*I/*RNase*A; 8) chromosomal DNA PCR; 9) negative control.

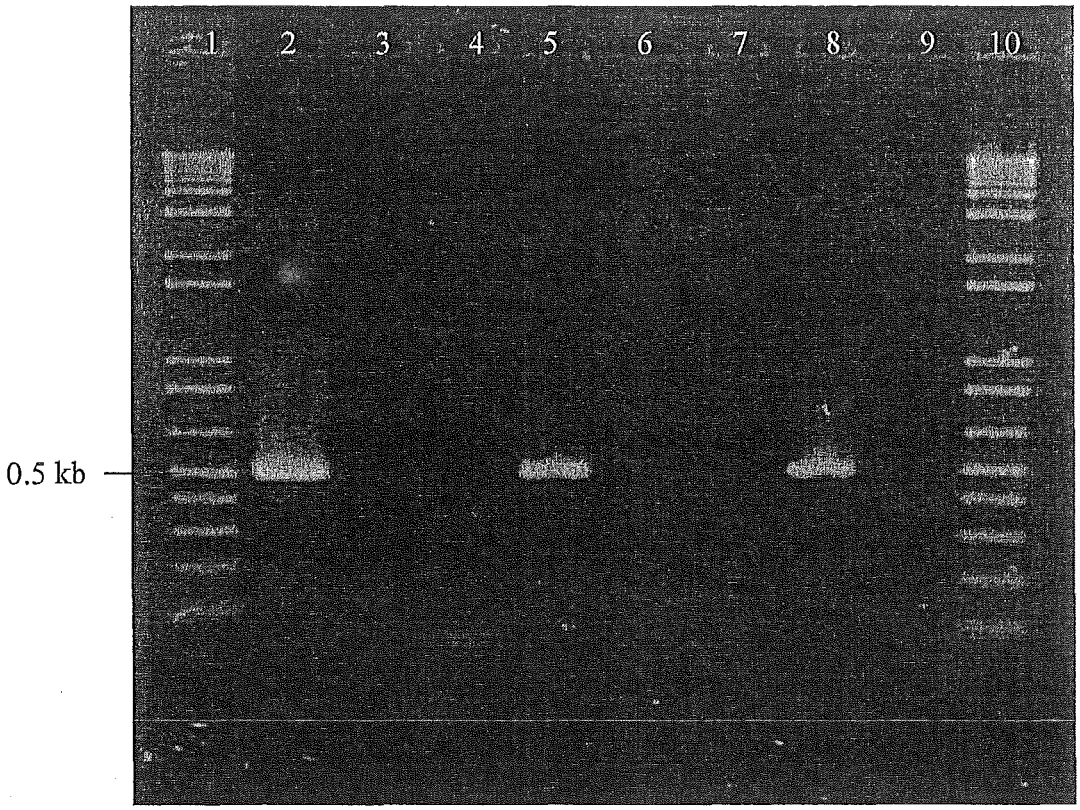


Figure 3.10. RT-PCR of *C. jejuni* 928 mRNA and *C. jejuni* NCTC11168 mRNA with 16S rDNA internal primers. Lanes: 1) and 10) 1 kb plus ladder; 2) 928 RNA treated with *DNAse*I; 3) 928 RNA treated with *RNase*A; 4) 928 RNA treated with *DNaseI/RNase*A; 5) NCTC11168 RNA treated with *DNaseI*; 6) NCTC11168 RNA treated with *RNase*A; 7) NCTC11168 RNA treated with *DNaseI/RNase*A; 8) chromosomal DNA PCR; 9) negative control.

All previous results suggested that *lex2B*, located between *gmhA* and *waaF* and present in single copy on the *C. jejuni* 928 possess information for synthesis of the putative Lex2B protein. The next step was to begin a functional study of the *lex2B* encoded protein. For that propose attempts to create a "null" mutant of lex2B on the *C. jejuni* 928 chromosome were made.

3.5 ALLELIC EXCHANGE OF *lex2B C. jejuni* 928

DNA sequence analysis showed that *lex2B* is physically connected to other genes involved in biosynthesis and assembly of the LPS core. Absence of a promoter sequence upstream of *lex2B* and *gmhA* suggested that transcription of these genes could be from the same promoter, located upstream of the *gmhA* gene. However, to determine the function of the Lex2B, it was necessary to create a *C. jejuni* 928 *lex2B* null mutant.

Comparison of LPS chemical structures (using mass spectroscopy) from *lex2B*$^+$ and *lex2B*$^-$ strains would provide information regarding the *lex2B* protein product.

3.5.1 CONSTRUCTION OF A SUICIDE PLASMID CONTAINING *Δlex2B*

The overall objective of this section was to determine if *lex2B* is in fact a glycosyl transferase. In order to achieve this, allelic exchange of *lex2B* from *C. jejuni* 928 with a plasmid allele, by single cross, homologous recombination was attempted. Plasmid pDJ4375 (Figure 3.11) was constructed such that an allele of *lex2B* lacking both 5' and 3' ends was located on the suicide plasmid pBIISK+. Additionally this plasmid was marked with a kanamycin resistance cassette specific for expression in *C. jejuni* (Konkel, 1999). As ColEl-based plasmids such as pBIISK+ cannot replicate in *C. jejuni*, kanamycin resistance can only be achieved if recombination onto the chromosome occurs. *E. coli* KLC4000 was transformed with the resulting plasmid, pDJ4375, and transformants were selected for kanamycin and ampicillin resistance. From 31 colonies showing resistance to these antibiotics, 16 were selected for verification of the presence of pDJ4375. Plasmids were reisolated and inserts were amplified by PCR using primers T7 and T3 (see Table 2.4). Only one transformant of the 16 produced the predicted amplicon of 2.1 kb (containing *Δlex2B* and the Km cassette) (data not shown). The plasmid that generated this amplicon was restriction digested with *Apa* I and *Pst* I to verify its size and structure (Figure 3.12). An ~5 kb fragment obtained from *Apa* I restriction digestion was expected since only one *Apa* I site is present in pDJ4375 (within the multiple cloning site of pBIISK+). The size of observed fragment was consistent with physical map of pDJ4375 (Figure 3.11). To verify the insert and to determine the orientation of *Δlex2B* in pDJ4375, the plasmid was digested with *Pst* I (Figure 3.12). Lengths of the fragments following *Pst* I digestion were also consistent with the pDJ4375 map. Once the structure of pDJ4375 was confirmed, this plasmid was used to transform electrocompetent *C. jejuni* 928 cells.

3.5.2 DETECTION OF A *C. jejuni* 928 *lex2B* MUTANT

Once inside *C. jejuni* 928, pDJ4375 must be recombined onto the chromosome if kanamycin resistance is to be conferred (Figure 3.13). No kanamycin-resistant transformants of *C. jejuni* 928 were recovered after using the method described in section 2.6.2 despite alteration of plasmid and kanamycin concentrations.

One explanation for these results is the poor transformation ability of *Campylobacter*. It is known that transformation of *Campylobacter* species by plasmid DNA or heterologous DNA happens at an extremely low frequency (Wang and Taylor, 1990). This frequency is low when either using electroporation (Miller *et al.*, 1988) or natural transformation as the method (Wang and Taylor, 1990). Time constraints prohibited continuation of these experiments for the thesis, however these experiments are still in progress with attempts to alter the protocol for preparing *C. jejuni* competent cells.

An alternative experiment was to insert a fully functional *lex2B* into a shuttle vector (pMEK180, see Table 2.4) and transform strain *C. jejuni* NCTC11168. Previous analyses suggested that this strain does not have information for synthesis of Lex2B protein. If that is the case *C. jejuni* NCTC11168 with an added copy of *lex2B* should alter the composition of LPS, detectable by mass spectrometry. SDS-PAGE analysis of LPS isolated from *C. jejuni* strains 928 and NCTC11168 showed that the LPS molecular mass differs between these two strains (data not shown). LPS from 928 has higher molecular mass than LPS isolated from NCTC11168, suggesting that 928 has at least one additional sugar in LPS. Serotyping, based on the Penner method (LPS used as heat-stable antigen) confirmed that differences between the LPS of these strains exists. *C. jejuni* 928 was identified as HS1 and *C. jejuni* NCTC11168 as HS2 serotype. The chemical structures of the LPS OS core from these serotypes revealed that GalNAc was present in HS1 as the last modified sugar in the nascent hexose chain, whereas in HS2 serotypes this sugar is missing from the OS core region. Presence of this sugar in HS1 is the only difference in chemical structure of core OS between serotypes HS1 and HS2. This difference suggests that the LPS from NCTC11168 containing a *lex2B* copy could change from HS2 to HS1 and show LPS of the same molecular mass as LPS from 928. To date the construction of shuttle vector containing the *lex2B* gene under a constitutive *Campylobacter* promoter is nearly complete.

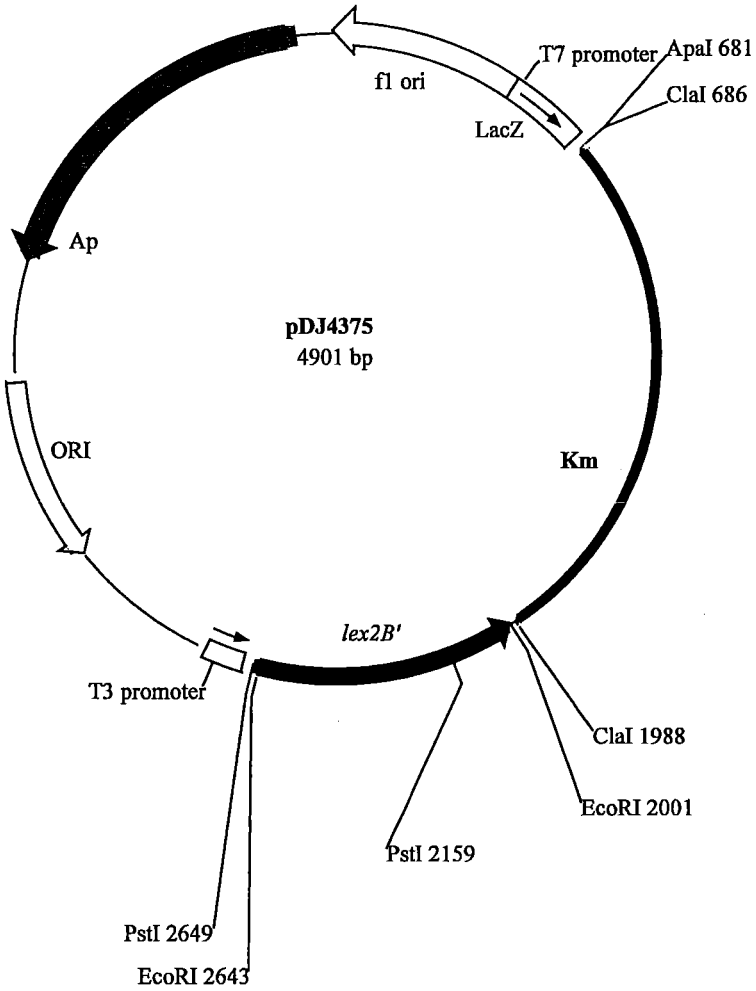


Figure 3.11. The restriction map of pDJ4375. pDJ4375 is estimated to be 4.9 kb, showing the orientation (solid black arrow) of the *C. jejuni* 928 truncated *lex2B* insert in pJK4370 and relevant restriction sites.

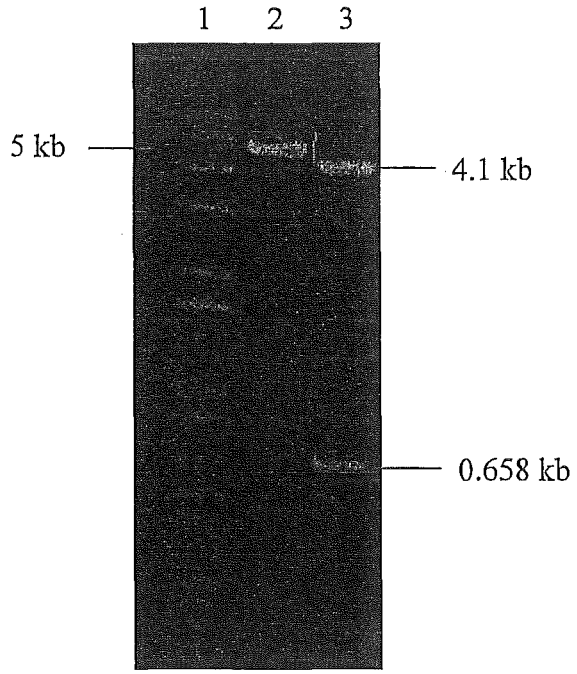


Figure 3.12. Restriction endonuclease digestion of pDJ4375. Lanes: 1) 1 kb plus ladder; 2) *Apa* I digestion; 3) *Pst* I digestion. Samples were electrophoresed through 0.8% agarose gel and visualised using ethidium bromide staining and UV light (see section 2.4.4).

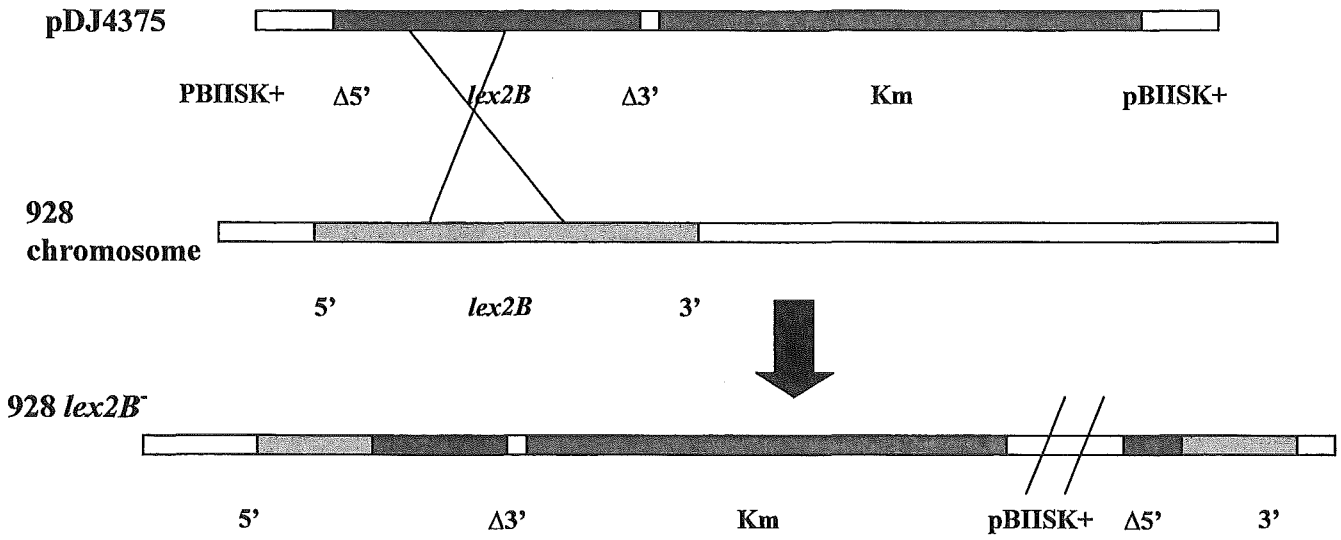


Figure 3.13. Allelic exchange of a truncated copy of *lex2B*. Through a single cross, homologous recombination event. Homologous recombination should only happen between the *lex2B* allele (orange rectangle) on the *C. jejuni* 928 chromosome and the truncated *lex2B* allele harboured on (red) pDJ4375. The resulting lex2B recombinant (928 *lex2B*⁻) would have mismatched 5' and 3' ends (eg., in one instance the 5' end will be provided by the plasmid allele and the 3' end from the chromosomal allele. **Abbreviations**: Km= kanamycin 200; pBIISK+= suicidal plasmid; // indicates that actual size of expression plasmid is 2.96 kb.

3.6 *lex2B* IN OTHER *C. jejuni* AND *C. coli* STRAINS

Yates (1998) determined, using PCR-RFLP analysis, that 5,85% of 105 analysed *C. jejuni* and *C. coli* isolates produce an ~1.7 kb amplicon when amplified with the oligonucleotide primers 96.01 and 94.293 (see Table 2.4). These isolates were classified as the *gmhA* 3 RFLP profile. To permit a wider examination of *lex2B* in other *C. jejuni* and *C. coli* strains, PCR, Southern and dot hybridisation analyses were applied. Strains from *gmhA* group 3(see Table 2.2), including *C. jejuni* strain 928, and NCTC11168 were amplified using primers 96.01 and 94.293. All strains (except NCTC11168) produced a 1.7 kb amplicon. After hybridisation of *lex2B* probe (see section 2.9.2) a signal was detected with all PCR products, again with the exception of NCTC11168 (Figure 3.14). These results suggested that it is likely that these strains possess *lex2B* on their chromosomes.

To confirm these results and to develop a rapid method for screening *Campylobacter* isolates for *lex2B*, dot blot hybridisation analysis was attempted (Figure 3.15). Chromosomal DNA (final concentration 10 ng/μl) from 17 bacterial strains were manually applied onto a nylon membrane and hybridised with the *lex2B* probe. Consistent with the previous hybridisation results (PCR amplicons), strains 928 and LG430I provided detectable signal. *C. coli* M275 DNA and the positive control, pDJ4216 were also positive. However, PCR positive but dot-blot negative strains (i.e., *C. jejuni* strains YP502F, MB617B, ZP028D and *C. coli* RC167B) suggests that this method is not completely operational. Unfortunately time constrains prevented a completion of this technique.

An alternative way to rapidly screen *C. jejuni* strains for the presence of *lex2B* was to create a monospecific polyclonal antibody for *lex2B* for use in a Western hybridisation experiment. In order to achive this Lex2B protein was expressed in *E. coli*.

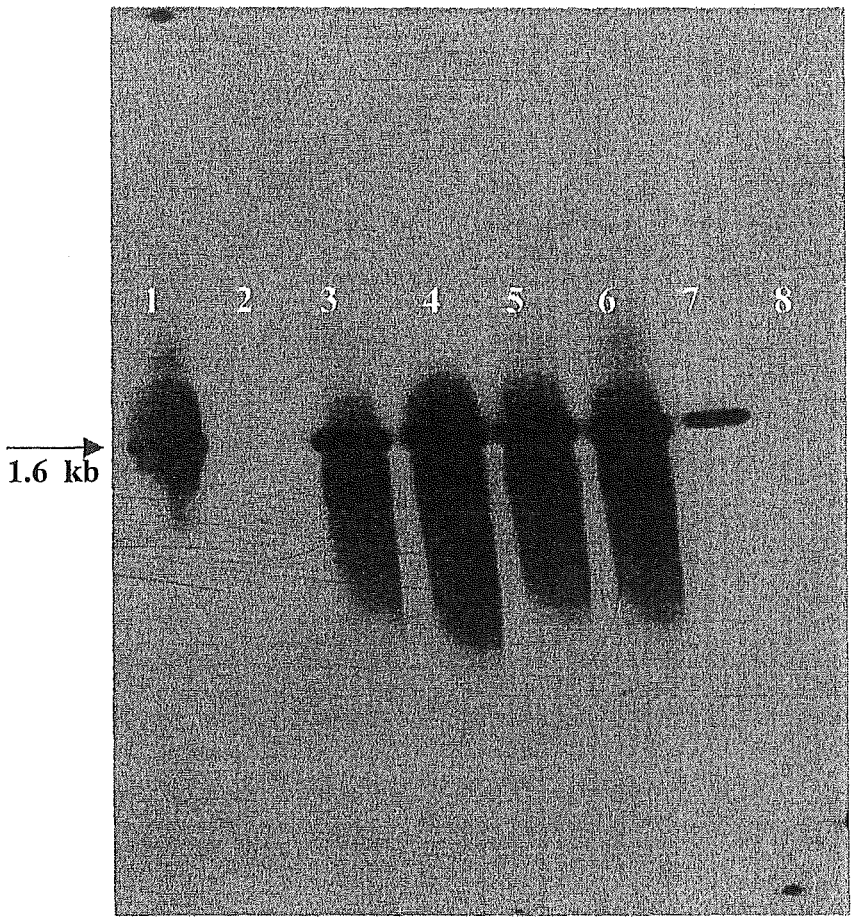


Figure 3.14. Southern hybridisation analysis of PCR products derived from chromosomal DNA of different *C. jejuni* and *C. coli* strains. Lanes: 1) *C. jejuni* 928; 2) *C. jejuni* NCTC11168; 3) *C. jejuni* YP502F; 4) *C. jejuni* MB617B; 5) *C. jejuni* LG430I; 6) *C. jejuni* ZP028D; 7) *C. coli* RC167B; 8) PCR negative.

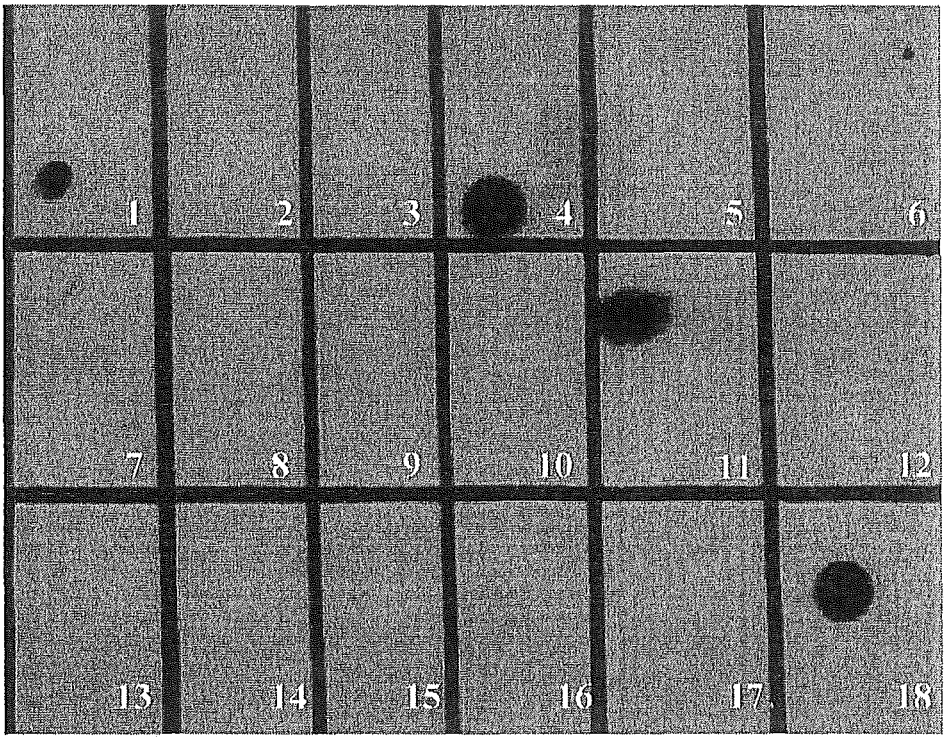


Figure 3.15. Detection of the *lex2B* gene in different bacterial strains. Dot-blot analysis was performed with the *lex2B* probe (see section 2.9). Lanes: 1) *C. jejuni* 928; 2) YP502F; 3) MB617B; 4) LG430I; 5) ZP028D; 6) KLC4303; 7) KLC4305; 8)KLC4297; 9) KLC4315; 10) *C. coli* RC167B; 11) *C. coli* M275; 12) NCTC11168; 13) F38011; 14) *E. coli* KLC4157; 15) *H. pylori*; 16) *Saccharomyces cerevisiae*; 17) *S. typhimurium*; 18) pDJ4216

3.7 Lex2B PROTEIN EXPRESSION

Expressing large amounts of protein from cloned genes introduced into *E. coli* has proven invaluable in the purification, localisation and functional analysis of proteins. To study the expression of *lex2B* and create a monospecific polyclonal antibody, pET vectors and *E. coli* strain KLC4166 (see Table 2.3, Table 2.1, section 2.8.1) were used.

The *Sst*I and *Xho*I restriction sites (GAGCT/C and C/TCGAG) in the multiple cloning cassette of pET24b were used to create an in frame fusion protein of Lex2B (see section 2.5). The resulting plasmid, pJKP030 (courtesy of J.D. Klena), contained a fusion of the *lex2B* gene with the initiation codon of the phage gene 10 leader sequence encoded in pET24b (Figure 3.16). The fusion gene was under the T7 promoter control. In

BL21(DE3)pLysS *E. coli*, T7 RNA polymerase is under control of pLysS plasmid which encodes T7 lysozyme, a natural inhibitor of T7 RNA polymerase. Upon IPTG induction, overproduction of T7 RNA polymerase renders low-level inhibition by T7 lysozyme. The T7 promoter is not recognised by the host cell RNA polymerases, so transcription of target gene is silent when no T7 RNA polymerase is present in the cell.

Plasmid pJKP030 was used to transform KLC4166, resulting in production of the new strain KLC4377. The plasmid was reisolated and digested again to verify its structure (data not shown). KLC4377 and two control strains KLC4166 and KLC4171 (see Table 2.1) were subjected to a time course expression experiment. KLC4171 contains the vector, pET11a, derived from the same origin as pET24b. This strain was used as a control for expression of vector encoded genes lacking the fusion protein. After growing until OD_{600} was ~0.4-0.6, production of the fusion protein was induced by adding IPTG to the cultures (see section 2.8.1). Results obtained after SDS-PAGE electrophoresis had shown that after a 30 minute IPTG induction, production of Lex2B began (Figure 3.17). Calculated molecular mass of this protein was approximately 27 kDa. After 5 hours an approximately 4.3 fold increase was witnessed in total protein concentrations (see Table 3.7.1). Total protein concentration measured after 24 h upon induction with IPTG did not shown further increases in Lex2B concentration. A possible explanation for this result is the saturation of the culture after overnight incubation. SDS-PAGE analysis of samples collected from uninduced and induced control cultures KLC4166 and KLC4171 did not show a protein band of 27 kDa, as expected, due to absence of fusion protein on pET11a (Figure 3.18).

This experiment shows that the *lex2B* was expressed in KLC4377 *E. coli* under T7 promoter transcription control. This fusion protein was further used to create a polyclonal antibody for *lex2B* by elution of protein, purification and then injection of a rabbit. At present, *lex2B* polyclonal antibody is under development in the laboratory of M. Konkel (Washington State University).

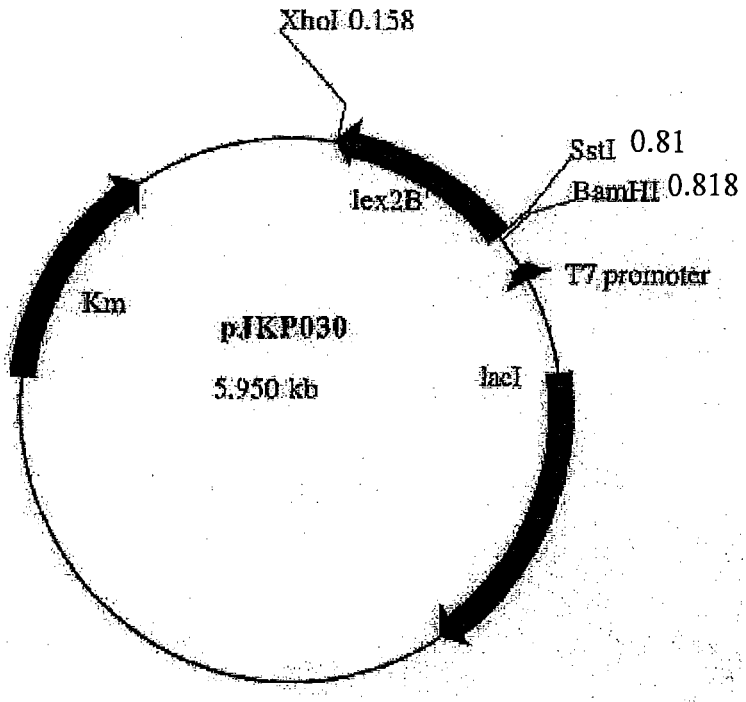


Figure 3.16. Physical map of pJKP030. pJKP030 is estimated to be 5.95 kb, showing direction of transcription (solid black arrows) and relevant restriction sites in pET24b.

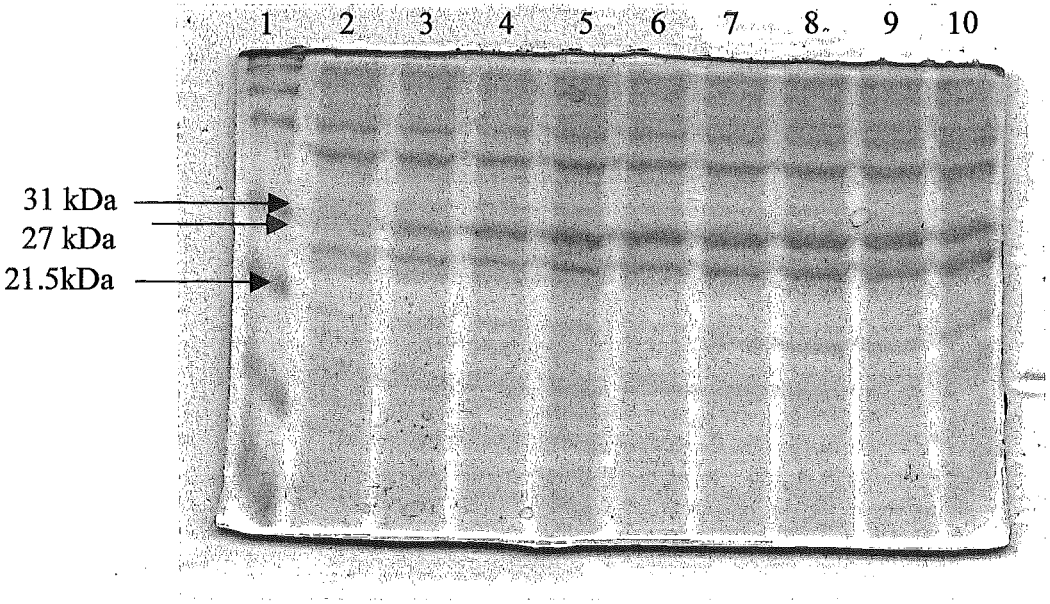


Figure 3.17. Electrophoretic analysis of protein extractions from KLC4377 (harbouring pJKP030) in IPTG induced time course experiment. Protein extractions were resolved in a SDS-15% polyacrylamide gel (SDS-PAGE) using tricine buffer system and visualised by Commassie Brilliant Blue staining as described in section 2.8.1. Lanes: **1)** low molecular weight marker; **2)** uninduced KLC4377; **3)** 30 min after IPTG induction; **4)** 60 min; **5)** 90 min; **6)** 120 min; **7)** 180 min; **8)** 4 h; **9)** 5 h; **10)** 24 h after induction.

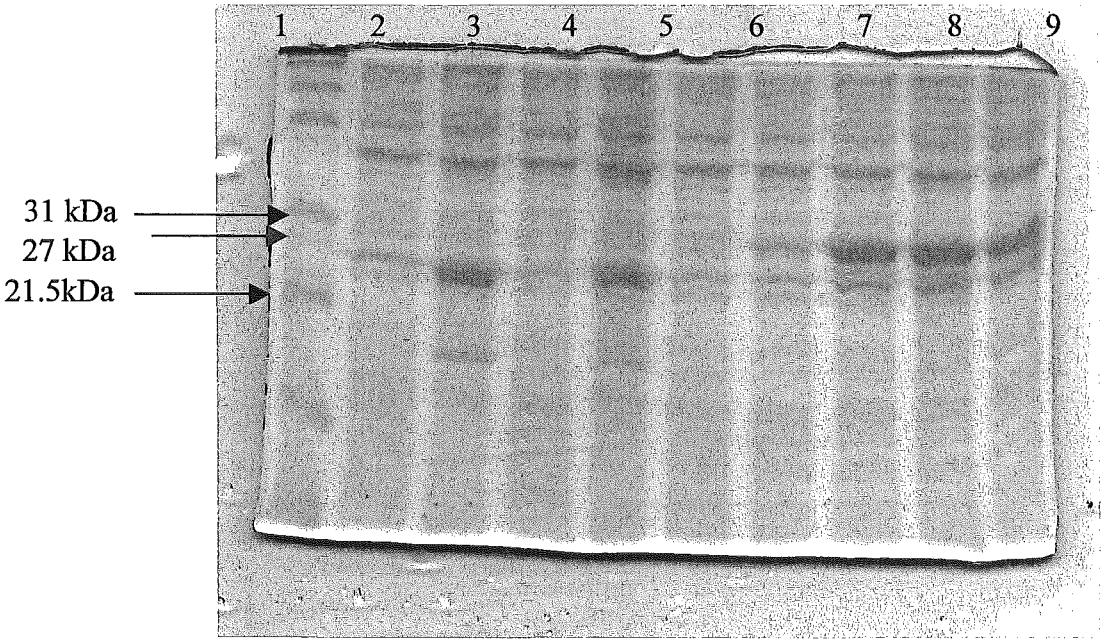


Figure 3.18. Electrophoretic analysis of protein extractions from KLC4166, KLC4171 (harbouring pET11a) and KLC4377. Proteins were resolved in a SDS-15% polyacryamide gel (SDS-PAGE) using a tricine buffer system and visualised by Commassie Brilliant Blue staining as described in section 2.8.1. Lanes: **1)** low molecular weight marker; **2)** KLC4166 uninduced; **3)** KLC4166 24 h upon IPTG induction; **4)** KLC4171 uninduced; **5)** KLC4171 24 h upon IPTG induction; **6)** KLC4377 uninduced; lanes **7,8,9,10)** KLC4377 30 min, 3 h, 5 h and 24 h after IPTG induction.

Table 3.2 Protein concentration measured by the method of Bradford.

strain	TIME								
	0'	60'	90'	120'	180'	3 h	4 h	5 h	24 h
KLC4166	0.358 *								2.148
KLC4171	0.544								2.154
KLC4377	0.446	0.947	1.230	1.394	1.574	1.802	1.927	2.038	2.055

* All concentrations are mg/ml

CHAPTER IV

DISCUSSION

In recent years *C. jejuni* has been an object of intensive research since it is a major cause of human gastroenteritis and a significant factor in cases of neurological disorders such as GBS and MFS (Salloway *et. al.*, 1996). LPS and LOS constituents of the outer membrane of Gram-negative organisms such as *C. jejuni* have been shown to be important virulence factors for causing infection. These molecules confer, amongst other things, serum resistance, antibiotic resistance and endotoxic properties on the bacterium (Rietschel and Brade, 1994). Studies involving other bacterial species suggest that antigenic and phase variation of LPS/LOS epitopes is an adaptive strategy for microbial evasion of host immune defensive systems. Whether this strategy is important for *C. jejuni* will be discussed below.

4.1 *lex2B* Shows Genomic Diversity

Upritchard (1997) established that the *C. coli* isolate M275 has an ORF located downstream from the *gmhA* gene that has significant amino acid sequence similarity with the *lex2B* gene from *H. influenzae*. This ORF was located between genes whose products are predicted to be involved in biosynthesis (*gmhA*) and assembly (*waaF*) of the LPS core region. In a separate study Yates (Yates, 1998), using *gmhA* as genetic marker to differentiate between *C. jejuni* and *C. coli* isolates, found that only a small percentage of human *C. jejuni* isolates might carry this gene. In this study I have isolated and characterised a *C. jejuni* strain 928 gene with a similar location to the *lex2B* gene in *C. coli*. Based on data generated in this thesis, I have subsequently named this gene *lex2B*. I have also shown, by PCR analysis using oligonucleotides specific for 23S rRNA and *cadF*, that 928 is indeed a *C. jejuni* isolate (Eyers *et. al.*, 1993; Konkel *et. al.*, 1999a).

Analysis of sequence data generated for a 1.7 kb PCR amplicon from *C. jejuni* 928 revealed the presence of two complete and one partial ORF. The first complete ORF of 561 nucleotides was capable of encoding a protein of 186 amino acids and had mol% (G+C) typical for *C. jejuni* genes (Nuijten *et. al.*, 1990). At the nucleotide level, this ORF showed little sequence similarity to the *gmhA* genes of *E. coli* and *H. influenzae*, but displayed 94% identity with *gmhA* from *C. jejuni* NCTC11168 and 86% with *gmhA* from *C. coli* M275. The predicted amino acid sequence displayed a greater degree of similarity to the *E. coli gmhA* gene, however. Conservation of the predicted GmhA protein among *C. jejuni* and *C. coli* species is extremely strong showing 98% identity between amino acid sequences. These results suggest that considerable nucleotide divergence between species is acceptable as long as the primary amino acid structure remains conserved (Dykhuizen, 1991). Analysis of interspecies-complementation (see section 3.1.2) and sequence data indicates that the first *C. jejuni* 928 gene contained within pDJ4216 is a functional homolog of the *E. coli gmhA* gene and encodes a product with glyceromannophosphate isomerase activity in *E. coli* K12.

Brooke *et al.* (1996) suggested the GmhA of *E. coli* is a cytosolic protein based on sequence analyses, but secondary structure prediction of GmhA from *C. coli* M275 revealed the presence of a transmembrane helix. This suggests that the protein is most likely membrane associated. A transmembrane helix within the GmhA protein was also reported for *Synechocystis* spp. (Kaneko *et. al.*, 1996).

A second ORF, downstream of the double termination codon of the *C. jejuni* 928 *gmhA*, contained 765 nucleotides capable of encoding 255 amino acids. Codon usage (data not shown) of deduced 255 amino acids and mol% (G+C) content showed strong AT richness (74.5%), with a high percentage of hydrophobic amino acids (e.g. Phe, Ile, Lys). This mol% (G+C) is not usual among other bacteria except in some pathogenicity islands (Alm *et. al.*, 1999). Considering that the whole *C. jejuni* genome is AT-rich (64%) this mol%(G+C) is not unusual. There are other functional genes in *C. jejuni* reported to have mol% (G+C) slightly under this typical level (e.g. *ciaB*). This suggests that the ORF have been recently acquired from another bacterial species by a horizontal transfer event. An example of a recent gene acquisition in *E. coli* demonstrating this

phenomenon is *waaS*, which has a significantly lower mol% (G+C) content than OS and KDO pathway genes (Brooke and Valvano, 1996).

At an amino acid level, the second ORF was shown to have a high degree of identity with Lex2B protein from *H. influenzae*. This homology was extremely strong in three regions (overall 74% similarity). Lex2B in *H. influenzae* is located in the *lex-2* locus. In this locus, it is transcribed along with a second gene, *lex2A*. The order of the two genes in the operon is *lex2A-lex2B*. Mutational analysis of the *lex-2* locus indicated that expression of *lex2B* is essential for synthesis of a specific LOS epitope. This is reflected in the fact that *lex2B*$^{+}$ strains react with monoclonal antibodies (MAb) 4C4 and 5G8. Extensive identity/similarity was observed with Lex2B of *H. pylori*. This is not suprising considering that *Helicobacter* has also AT rich genome.

Genes from other bacteria that showed a certain degree of similarity with the putative *lex2B* from *C. jejuni* 928 (i.e., *lob1*, *lgtB*, *lgtE*, *lic2B*) are almost exclusively glycosyl transferases involved in biosynthesis of OS outer domain of LPS/LOS. These genes are also phase variable. HCA analysis revealed that *lex2B* has a motif, $D^{30\text{-}40}EDD^{90\text{-}110}$ (where the numbers refer to the location of amino acids in the polypeptide sequence) characteristic for processive sugar transferases. Comparison between nucleotide and amino acid sequences of *lex2B* from *C. jejuni* 928 and *C. coli* M275 revealed divergence that is unexpected for what appears to be an homologous gene in two species from the same genus. The amino acid region near the C-terminus of the *lex2B* gene product is especially variable, suggesting that this part of gene is a place of frequent recombination. This divergence also suggests that *lex2B* is an acquired gene from another bacterial species. Differences in nucleotide sequence could reflect either multiple acquisition events or species-specific changes that have occurred over time.

Southern hybridisation experiments determined that the *C. jejuni* 928 *lex2B* gene is present on the chromosome as a single copy. The same analysis confirmed previous speculation, based on nucleotide sequencing and PCR-RFLP typing, that *C. jejuni* NCTC11168 is without *lex2B*. Absolute conformation of the absence of *lex2B* in *C. jejuni* NCTC11168 has been provided by the complete genome sequence (Sanger centre, 1998). The *lex2B* nucleotide sequence was compared with the genomic sequence of

NCTC11168 and no significant similarities/identities were uncovered. This suggests that appearance of *lex2B* is not the rule for *C. jejuni* isolates.

The third gene transcribed in an opposite orientation from *gmhA* and *lex2B* and which overlaps the putative *lex2B* sequence has been shown to contain a functional homolog of the *S. typhimurium waaF* (heptosyl transferase II) gene. This arrangement of genes, *gmhA-lex2B-waaF*, has only been reported for *C. coli* M275 to date, thus this arrangement appears to be *Campylobacter* specific. However, in *C. jejuni* strains missing the *lex2B* gene, *gmhA* is physically linked with *waaF*, based on previous sequence analyses. Further, organisation of *gmhA* appears to be different depending upon the organism studied. Many of the genes necessary for LPS core biosynthesis in *E. coli* are located in the large *waa* operon at 81 minutes on chromosome (Schnaitman and Klena, 1993). The genes *gmhD*, *waaF* and *waaC* which are required for biosynthesis and attachment of heptose are in one region of the cluster. The *E. coli* K12 *gmhA* has been reported to be physically unlinked to this cluster, mapping at 5.3 minutes on the chromosome (Brooke and Valvano, 1996). GmhA is linked in *H. ducreyi* and *H. influenzae* with genes involved in dipeptide (*dpp*) transport and arginine uptake (*apt*). Finally, it is interesting to speculate on the organisation of *waaF* in relationship to *gmhA* and *lex2B* genes. Perhaps the organisation of these genes in a tail to tail fashion represents a means of genetic regulation of heptose attachment/biosynthesis of the LPS core, the presence of larger operons allowing expression in concert.

To develop a rapid method for screening large numbers of *C. jejuni* isolates for the presence of *lex2B*, dot-blot analysis was used. Results were inconsistent; all isolates that were shown to amplify a 1.7 kb amplicon by PCR (Yates, 1998) and to contain *lex2B* by probe hybridisation to these PCR amplicons (this study) did not always yield a positive result in the dot blot analysis. Possible explanations for these inconsistencies are poor application of genomic DNA to the nylon membrane or concentrations of genomic DNA were too low to be detected by this method. Unfortunately time constraints did not permit full development of this method. However it is clear that *lex2B* is present in only a small percentage of *C. jejuni* strains. A recently reported gene, *orfE*, involved in O-chain biosynthesis of *C. jejuni* NCTC11168 was also found to present in only a small

percentage of *C. jejuni* strains, independent of serotype (Wood *et. al.*, 1999). Mutation analyses of this gene had no detectable effect on LOS phenotype in NCTC11168 but did affect, when added to *C. jejuni* NCTC11828, O-chain production within the LPS. This raises the speculation that increasing rates of human infection with *C. jejuni* could be connected with changes in LPS.

4.2 REGULATION OF *lex2B* EXPRESSION

As stated above, the *lex-2* locus in *H. influenzae* encodes two genes, *lex2A* and *lex2B*. The promoter for this locus is situated 114 bp upstream of the *lex2A* 5' end. The *lex2A* contains 18 tandem repeats of the nucleotide tetramer *GCAA* near its 5' end. The presence of these repeats provides the opportunity for slipped-strand mispairing, which alters the number of tandem repeats and thereby affects expression of these genes. Subsequently, the LOS epitopes reactive with MAb 4C4 and 5G8 are altered in such a fashion that these MAbs are no longer reactive. The presence of the LOS epitope reactive with MAb 5G8 has been correlated with virulence expression in some strains of *H. influenzae*. Mutation analysis has not shown if *lex2A* is required for expression of *lex2B* (Jarosik and Hansen, 1994). Nucleotide sequencing of the putative *lex2B* from *C. jejuni* 928 has not revealed the presence of repeated tandem residues within itself of within the *gmhA* gene suggesting that *lex2B* must have different regulation system than the one reported for *lex2B* from *H. influenzae*.

Inspection of the nucleotide sequence upstream of the *gmhA* initiation site and within *gmhA* in pDJ4216 failed to reveal potential –35 and –10 consensus promoter sequences for *lex2B* transcription. This was not unexpected as the *gmhA* and *lex2B* nucleotide sequences overlap, suggesting that they may be cotranscribed. While no promoter was located upstream of *gmhA* either, analysis of the nucleotide sequence from NCTC11168 suggests that a promoter may be located further upstream (Vasan, D., M.Sc. thesis, 1999). Current research in our laboratory indicates that promoter sequence for *gmhD-gmhB/C-gmhA* is upstream of *gmhD*. Considering that *lex2B* is transcriptionaly and physically connected with *gmhA*, I speculate that this promoter also regulates transcription of *lex2B*. Investigation of possible transcriptional and translation

connection of *lex2B* with upstream genes *gmhA*, *gmhB/C* and *gmhD*, using RT-PCR did not show that these genes are transcribed from the same promoter (see section 3.4) Further experiments using more appropriately designed primers are necessary to assess this issue in more detail.

4.3 EXPRESSION ANALYSES OF *lex2B*

In recent years, the ability to produce and quantitate mRNA has become invaluable as a tool to monitor gene expression. To assess expression of the *lex2B* gene, production of mRNA was monitored. Total RNA isolated from *C. jejuni* 928 and *C. jejuni* NCTC11168 was digested with *DNase*I to eliminate any traces of DNA that could create a false positive with RT-PCR. Success of this treatment was indicated by the absence of amplified products in *DNase*I-treated controls. No RT-PCR-amplified products were observed in *RNase*A-treated samples, verifying that the target nucleic acid was RNA. This result suggests the *lex2B* gene is likely to be functional since it is transcribed into mRNA, which results in production of protein. Absence of an amplification signal from *lex2B* in *C. jejuni* NCTC11168 supplies further proof that this strain does not contain this gene.

Once mRNA from the *lex2B* gene was detected, the gene was placed under phage T7 promoter control. Under transcriptional control of the T7 promoter, overexpression of the Lex2B protein was achieved. The detected fusion protein was 27 kDa. However, the only conclusion to be drawn from this result is that Lex2B protein may be expressed in *E. coli*; it does not provide any information if Lex2B protein is expressed *in vivo* in *C. jejuni*. This experiment does permit generation of a monospecific polyclonal antibody. The polyclonal *lex2B* antibody is an effective tool for screening *C. jejuni* isolates for presence of this product and can provide a picture, over time, about how the percentage of isolates containing this gene product changes. This antibody can also be used to determine if *lex2B* expression is connected with certain HS (Penner) serotypes.

Generally, at the organism level, isolates of *C. jejuni* show considerable heterogeneity in the outer LPS core (Aspinall *et. al.*, 1993b). It is tempting to speculate that different

levels of gene expression as a result of changes in gene order with recently acquired genes (like *lex2B*) and presence of transcriptional coupling to adjacent genes could provide a mechanism for this variation.

4.4 FUNCTION OF *lex2B* IN *C. jejuni* 928

Serotyping using the Penner method determined that *C. jejuni* 928 is a member of the HS1/HS44 serogroup (the antigen giving higher titer was listed first). This method identifies over 60serotypes (at least 44 *C. jejuni* and 18 *C. coli*) using LPS as a heat stable antigen. Aspinall *et al.* (1992, 1993a, 1993b, 1993c, 1994, 1995) characterised the chemical structure of LPS from serotypes HS1, HS2, HS3, HS4, HS19, HS23 and HS36. Knowing that *C. jejuni* NCTC11168 is a member of the HS2 serogroup and *C. jejuni* 928 is HS1, I speculated from the known LPS chemical structures of these two serotypes that the role of the Lex2B protein in LPS biosynthesis could be a galactosyl transferase. As Figure 4.1 shows the only difference between the two HS types appears to be the presence of GalNAc linked (α1-4) to the last Gal molecule in nascent OS outer core chain. If the *lex2B* gene product is a sugar transferase as suggested by hydrophobic cluster analysis then it is possible that its role is the transfer of GalNAc to the outer domain of OS. Similar to HS1, serogroups HS3, HS23 and HS36 also possess GalNAc as the last molecule in the LPS chain. Additionally the OS structure of these serotypes strongly resembles the human ganglioside G_{M2}. If *lex2B* is responsible for the transfer of this molecule, it is expected that *lex2B* is present on chromosome of other serotypes containing this sugar epitope. Further to this PCR analysis using *lex2B* primers of HS23 and HS36 *C. jejuni* strains showed that it is likely that these serotypes contains *lex2B* since they produce PCR amplicons with *lex2B* specific primers (data not shown).

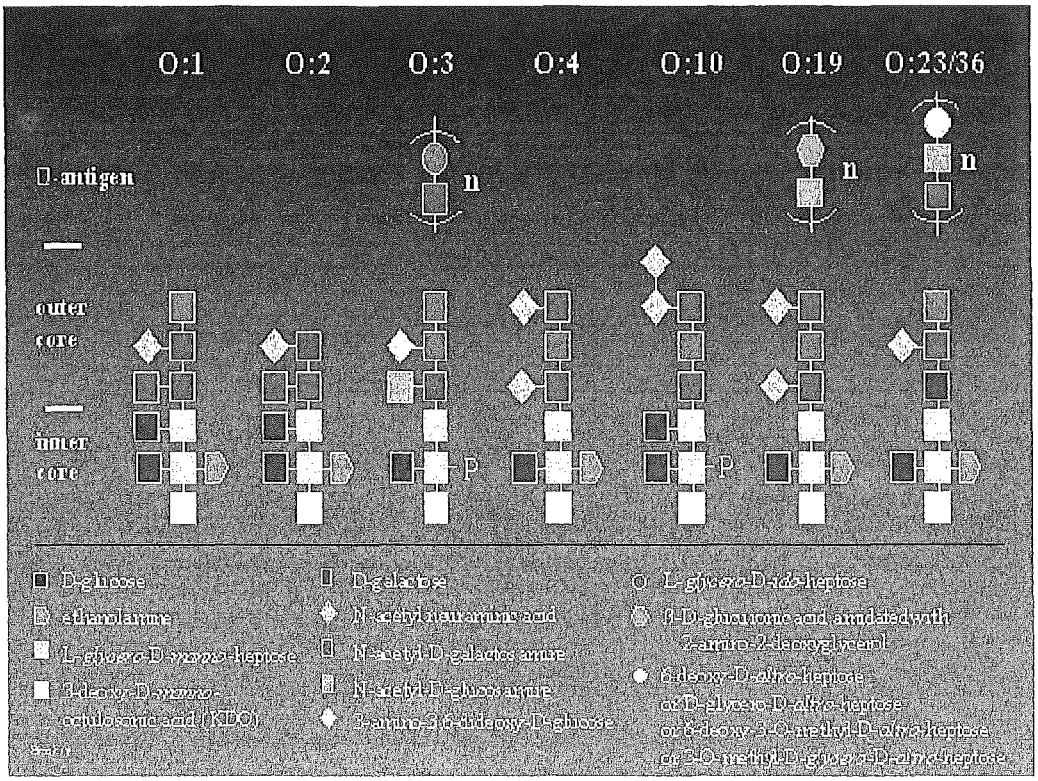


Figure 4.1. Known structure of core molecules and O antigen-like polysaccharides from *C. jejuni* serotypes that contain potential cross-reactive epitopes with different gangliosides. (Adapted from Nachamkin, 1998).

To assess the exact function of *lex2B* in *C. jejuni* 928, attempts to create a "null" mutant were made. A plasmid construct carrying the truncated *lex2B* with kanamycin as a marker was engineered, but to date all attempts to obtain *C. jejuni* 928 (*lex2B*$^-$) kanamycin resistant isolates have failed. As mentioned previously, one possible explanation for this is the poor transformation capacity of *C. jejuni*. The method used to transform *C. jejuni* cells with a truncated *lex2B* allele carried on a suicide plasmid has been shown to be successful in recent studies (Konkel *et. al.*, 1997). Alteration of the protocol for preparing competent *C. jejuni* cells is under way. At the same time a different approach was taken, using a shuttle plasmid containing a *Campylobacter* origin of replication and *lex2B* to transform *C. jejuni* strains showing absence of *lex2B* on their chromosomes (e.g. *C. jejuni* NCTC11168). Addition of *lex2B* in these strains should change the LPS structure visible on SDS-PAGE gel and also change serotype (i.g., *C. jejuni* NCTC11168 HS2 would change to HS1). This experiment is also under way.

4.5 CONCLUSIONS

In this thesis the isolation and characterisation of the *C. jejuni lex2B* gene from strain 928 has been described. This gene appears to encode a galactosyl transferase that may be involved in the transfer of GalNAc to the last Gal molecule in the LPS chain.

The *gmhA* gene is highly conserved among *C. jejuni* and *C. coli* isolates. In a small percentage of these isolates *gmhA* is physically and possibly transcriptionally linked with novel gene, *lex2B*. Codon usage and mol% (G+C) content suggest *lex2B* has been acquired recently by *Campylobacter* isolates. This gene is a variable feature of the *Campylobacter* spp. genomic landscape.

Expression of the lex2B gene results in a protein of calculated molecular mass of 27 kDa. HCA analysis revealed that the *lex2B* gene product has an amino acid motif characteristic for other galactosyl transferases.

In other enteric and non-enteric bacteria, *lex2B* is essential for synthesis of LPS/LOS epitopes. The regulation of *lex2B* in these organisms is probably through slip strand mispairing mechanism. Based on the nucleotide sequence of *lex2B* and adjoining sequences, regulation in *C. jejuni* does not appear to be through this type of mechanism. The speculation that *lex2B* is a sugar transferase involved in attachment of the last modified sugar, GalNAc, to the outer domain of OS remains to be determined as outlined previously.

4.6 FUTURE EXPERIMENTS

Future experiments include assessing the expression of *lex2B* over the cell cycle, in order to determine if antigenic variation due to Lex2B presence/absence occurs at the isolate level. For this purpose mRNA will be isolated from *C. jejuni* 928 at different growth densities and subjected to RT-PCR for the *lex2B* mRNA. Additionally, the level of Lex2B protein can be monitored by Western blot analysis. Design of appropriate primers spanning a smaller DNA fragment (i.e., forward primer in *gmhA* near the 3'end and reverse primer near 5' end of *lex2B*) should clear the picture regarding the

transcription of these two genes. Finally, binding and internalisation assays are necessary to define the effect changes in LPS structure caused by *lex2B* have on the virulence of *C. jejuni*. These experiments will initially be done in a cell culture assay, and if promising, moved to a live animal model (e.g., chicks).

REFERENCES

Allos, B.M and Blaser, M.J (1995). *Campylobacter jejuni* and the expanding spectrum of related infections. Journal of Infectious Diseases, vol. 20, pp 1092-1101.

Alm, R.A. (1999). Genomic-sequence comparation of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature, vol. 397, pp 176-180.

Altekruse, S.F., Hunt, J.M., Tollefson, L.K. and Madden, J.M. (1994). Food and animal sources of human *Campylobacter jejuni* infection. JAVMA, vol. 204, no. 1, pp 57-61.

Aspinall, G.O., McDonald, A.G., Raju, T.S., Pang, H., Mills, S.D., Kurjanczyk, L.A. and Penner, J.L. (1992). Serological diversity and chemical structures of *Campylobacter jejuni* low molecular weight lipopolysaccharides. Journal of Bacteriology, vol. 174, pp 1324-1332.

Aspinall, G.O., McDonald, A.G., Raju, T.S. Pang, H. Kurjanczyk, L.A. Penner, J.L. and Morran, A.P. (1993a). Chemical structure of the core region of *Campylobacter jejuni* serotype O:2 lipopolysaccharide. Europian Journal of Biochemistry, vol. 213, pp 1029-1037.

Aspinall, G.O., McDonald, A.G., Raju, T.S. Pang, H. Morran, A.P. Penner, J.L. (1993b). Chemical structure of the core region of *Campylobacter jejuni* serotype O:1, O:4, O:23 and O:36 lipopolysaccharides. Europian Journal of Biochemistry, vol. 213 pp 1017-1027

Aspinall, G.O., Fujimoto, S., Mcdonald, A.G., Pang, H., Kurjanczyk, L.A. and Penner, J.L. (1994a). Lipopolisaccharides from *Campylobacter jejuni* associated with Guillian-Barre syndrome patients mimic human gangliosides in structure. Infection and Immunity, vol. 62, no. 5, pp 2122-2125.

Aspinall, G.O., Fujimoto, S., Mcdonald, A.G., Pang, H., Kurjanczyk, L.A. and Penner, J.L. (1994b). Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: Structures of core oligosaccharide regions from the serostrain and two bacterial isolates from patients with the Guillain-Barre syndrome. Biochemistry, vol. 33, pp 241-249.

Aspinall, G.O., McDonald, A.G., Pang, H. (1994c). Lipopolisaccharide of *Campylobacter jejuni* serotype O:19; structures of O-antigen chains from the serostrain and two bacterial isolates from patients with the Guillian-Barre syndrome. Biochemistry, vol. 33, pp 250-255.

Aspinall, G.O., Lynch C.M., Pang, H., Shaver R.T. and Moran A.P. (1995). Chemical structure of the core region of *Campylobacter jejuni* O:3 lipopolisaccharide and an associated polysaccharide. Europian Journal of Biochemistry, vol. 231, pp 570-578.

Autenrieth, I.B., Schwarzkopf, A., Ewald, J.H., Karch, H. and Lissner, R. (1995). Bactericidal properties of *Campylobacter jejuni*-specific immunoglobulin M antibodies in commercial immunoglobulin preparations. Antimicrobial Agents and Chemotherapy, vol. 39, pp 1965-1969.

Bachman, B.J. (1990). Linkage map of *Escherichia coli* K12, edition 8. Microbiological Reviews, vol. 54, pp 130-197.

Belunis, C.J. and Raetz, C.R.H. (1992). Biosynthesis of Endotoxins. The Journal of Biological Chemistry, vol. 267, no. 14, pp 9988-9997.

Belunis, C.J., Clementz, T., Carty, S.M. and Raetz, C.R.H. (1995). Inhibition of lipopolysaccharide biosynthesis and cell growth following inactivation of the *kdtA* gene in *Escherichia coli*. The Journal of Biological Chemistry, vol. 270, no. 46, pp 27646-27652.

Black, R.E., Levine, M.M., Clements, M.L., Hughes, T.P. and Blaser, M.J. (1988). Experimental *Campylobacter jejuni* infection in humans. The Journal of Infectious Disease, vol. 157, pp 472-478.

Blaser, M.J. and Reller, L.B., (1981). *Campylobacter* enteriris. The New England Journal of Medicine, vol. 305, pp 1444-1452.

Blaser, M.J., Taylor, D.N. and Feldman, R.A. (1983). Epidemiology of *Campylobacter jejuni* Infections. Epidemiologic Reviews, vol. 5, pp 157-177.

Bourke, B., Sherman, P., Louie, H., Hani, E., Islur, P. and Chan, V.L. (1995). Physical and genetic map of the genome of *Campylobacter upsaliensis*. Microbiology, vol. 147, pp 2417-2424.

Brooke, J.S. and Valvano, M.A. (1996). Biosynthesis of inner core lipopolisaccharide in enteric bacteria, identification and characterization of a conserved phosphoheptose isomerase. Journal of Bacteriology, vol. 178, pp 3339-3341.

Burnes, A., Stucki, U., Nicolet, J. and Frey, J. (1995). Identification and characterisation of an immunogenic outer membrane protein of *Campylobacter jejuni*. Journal of Clinical Microbiology, vol. 33, no.11, pp 2826-2832.

Callebaut, I., Labesse, G., Durand, P., Poupon, A., Canard, L.,Chomilier, J., Henrissat, B. and Mornon, J.P. (1997). Cell Moleculare Life Science 53, 621-645.

Castillo, A. and Escartin, E.F. (1994). Survival of *Campylobacter jejuni* on sliced watermelon and papaya. Journal of Food Protection, vol. 57, no.2, pp 166-168.

Chang, N. and Taylor, D.E. (1990). Use of Pulsed-Field Agarose Gel Electrophoresis to size genomes of *Campylobacter* species and to construct a *Sal* I map of *Campylobacter jejuni* UA580. Journal of Bacteriology, vol. 172, no. 9, pp 5211-5217.

Chen, L. and Coleman Jr, W.G. (1993). Cloning and characterisation of the *Escherichia coli* K12 *rfa*-2 (*rfaC*) gene, a gene required for lipopolisaccharide inner core synthesis. Journal of Bacteriology, vol. 175, pp 2534-2540.

Coleman, J. and Raetz, C. R. H. (1988). First committed step of lipid A biosynthesis in *Escherichia coli*: Sequence of the *lpx* A gene. Journal of Bacteriology, vol. 170, no. 3, pp 1268-1274.

Conrad. R.S. and Galanos, C. (1990). Characterization of *Campylobacter jejuni* lipopolysaccharide. Current Microbiology, vol. 21, pp 377-379.

Constantinidou, C., Hellyer, T.J., Richardson, P.T. and Penn, C.W. (1996). Cross-reactivities and strain specificity of monoclonal antibodies to *Campylobacter jejuni* and *Heliobacter pylori* flagellins. In *Campylobacter*, *Heliobacter* and related organisms. Edited by Newell, D.G., Ketley, J.M. and Feldman, R.A.. New York: Plenum Press.

Cope, L.D., Yogev, R., Mertsola, J., Latimer, J.L., Hanson, M.S., McCracken, G.H. and Hansen, E.J. (1991). Molecular cloning of a gene involved in lipooligosaccharide biosynthesis and virulence expression by *Haemophilus influenzae* type b. Molecular Microbiology, vol. 5, pp 1113-1124.

Corry, J.E.L., Post, D.E., Colin, P. and Laisney, M.J. (1995). Culture media for the isolation of Campylobacters. International Journal of Food Microbiology, vol. 26, pp 43-76.

Cover, C. and Blaser, M.G. (1989). The patology of *Campylobacter infections* in humans. Annual Review of Medicine, vol. 40, pp 269-285.

Cundell, D.R and Tuomanen, E. (1995). Attachement and interaction of bacteria at respiratory mucosal surfaces. Virulence mechanisms of Bacterial Pathogens, 2nd edition, Edited by Roth, J.A *et.al.* American Society for Microbiology, Washington DC, pp 3-20.

Daniels, D.L., Plunkett III, G., Burland, V. and Blattner, F.R. (1992). Analysis of the *Escherichia coli* genome: DNA sequence of the region from 84.5 to 86.5 minutes. Science, vol. 257, pp 771-778.

De Melo, M.A. and Peche're, J-C. (1990). Identification of *Campylobacter jejuni* surface proteins that bind to eucariotic cells *in vitro*. Infection and Immunity, vol. 58, pp 1749-1756.

Defives, C.R., Bouslamti, J.C., Derieux, O. and Fournet, B. (1989). Characterisation of sialic acids containing lipopolisaccharide from *Rhizobium meliloti*. FEMS Microbiol. Letters, vol. 57, pp 203-208.

Doig. P., Yao. R., Burr, D.H., Guerry, P., and Trust, T.J. (1996). An environmentally regulated pilus-like appendage involved in Campylobacter pathogenesis. Molecular Microbiology, vol. 20 no. 4, pp 885-894.

Eberhart-Phillips, J., Walker, N., Garrett, N., Bell, D., Sinclair, D., Rainger, W. and Bates, M. (1995). Campylobacteriosis in New Zealand – Results of the MAGIC study. Prepared on contract to the Ministry of Health and the Public Health Commission.

Eidels, L. and Osborn, M.J. (1971). Lipopolysaccharide and aldoheptose biosynthesis in transketolase mutants of *Salmonella typhimurium*. Proceedings of the National Academy of Sciences USA vol. 68, pp 1673-1677.

Eidels, L. and Osborn, M.J. (1974). Phosphoheptose Isomerase, First enzyme in the biosynthesis of aldoheptose in *Salmonella typhimurium*. The Journal of Biological Chemistry, vol. 249, pp 5642-5648.

Erwin, A.L., Haynes, P.A., Rice, P.A. and Gotchlich, E.C. (1996). Conservation of the lipopolisaccharide synthesis locus *lgt* among strain of *Neisseria gonorrhoeae*: requirement for *lgtE* in synthesis of the 2C7 epitope and of the β chain of strain 15253. Journal of Experimental Medicine, vol. 184, pp 1233-1241.

Eyers, M., Chapelle, S., van Camp, G., Goossens, H. and de Wachter, R. (1993). Discrimination among thermphilic *Campylobacter* species by polymerase chain reaction amplifications of 23S rRNA gene fragments. Journal of Clinical Microbiology, vol. 31, pp 3340-3343.

Faoagali, J.L. (1984). *Campylobacter* in New Zealand. New Zealand Medical Journal, vol. 97, pp 560-561.

Field, L.H., Headley, V.L., Payne, S.M. and Berry, L.J. (1986). Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. Infection and Immunology, vol. 54, pp 126-132.

Field, L.H., Underwood, J.L., Payne, S.M., and Berry, L.J. (1993). Characteristics of an avirulent *Campylobacter jejuni* strain and its virulence-enhanced variants. Journal of Medical Mcrobiology, vol. 38. pp 293-300.

Gamian, A., Romanowska, A. and Romanowska, E. (1992). Immunochemical studies on sialic acid-containing lipopolisaccharides from enterobacterial species. FEMS Microbiol. Immun. 4:pp 323-328.

Garvis, S.G., Puzon, G.J. and Konkel, M.E. (1996). Molecular Characterisation of a *Campylobacter jejuni* 29-Kilodalton Periplasmic Binding Protein. Infection and Immunity, vol. 64, no. 9, pp 3537-3543.

Goldman, R.,Kohlbrenner, W., Lartey, P. and Pernet, A. (1987). Antibacterial agents specifically inhibiting lipoplysaccharide synthesis. Nature, vol. 329, pp 162-164

Guerry, P., Doig, P., Alm, R.A., Burr, D.H., Kinsella, N. and Trust, T. (1996). Identification and characterisation of genes required for posttransitional modification of *Campylobacter coli* VC167 flagellin. Molecular Microbiology, vol. 19, pp 369-378.

Hammond, S.M., Claesson, A., Jansson, A.M., Larsson, L.G., Pring, B.G., Town, C.M. and Ekstrom, B. (1987). A new class of synthetic antibacterials acting on lipopolysaccharide biosynthesis. Nature, vol. 327, pp 730-732.

Heinrichs, D.E., Monteiro, M.A., Perry, M.B. and Whitfield, C. (1998a). The assembly system for the lipopolisaccharide R2 core-type of *Escherichia coli* is a hybrid of those found in *Escherichia coli* K-12 and *Salmonella enterica*. The Journal of Biological Chemistry, vol. 273, no 15, pp 8849-8859.

Heinrichs, D.E., Yethon, J.A., Amor, P.A. and Whitfield, C. (1998b). The assembly system for the outer core portion of R1-and R4-type lipopolisaccharides of *Escherichia coli*. The Journal of Biological Chemistry, vol. 273, no. 45, pp 29497-29505.

Hitchcock, P.J. and Brown, T.M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver stained polyacrylamide gels. Journal of Bacteriology, vol. 154, pp 269-277.

Hood, D.W., Deadman, M.E., Allen, T. (1996). Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolisaccharide biosynthesis. Molecular Microbiology, vol. 17, pp 1167-1175.

Hugdahl, M.B., Beery, J.T. and Doyle, M.P. (1988). Chemotactic behaviour of *Campylobacter jejuni*. Infection and Immunology, vol. 56, pp 1560-1566.

Inzana, T.J., Hensley, J., McQuiston, J., Lesse, A.J., Campagnari, A.A., Boyle, S.M. and Apicella, M.A. (1997). Phase variation and conservation of lipopolysaccharide epitopes in *Haemophilus somnus*. Infection and Immunity, vol. 56, no. 11, pp 4675-4681.

Jarosik, G.P. and Hansen, E.J. (1994). Identification of a new locus involved in expression of *Haemophilus influenzae* type b lipopolysaccharide. Infection and Immunity, vol. 62, pp 4861-4867.

Jenings, M.P., Hood, D.W., Peak, I.R.A., Virji, M. and Moxon, E.R. (1995). Molecular analysis of a locus for the biosynthesis and phase-variable expression of the lact-N-neotetraose terminal lipopolisaccharide structure in *Neisseria meningitidis*. Molecular Microbiology, vol. 18, pp 729-740.

Jiang, X.M., Neal, B., Santiago, F., Lee, S.J., Romona, L.K. and Reeves, P.R. (1991). Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). Molecular Microbiology, vol. 5, pp 695-713.

Joiner, K.A. (1985). Studies on the mechanism of bacterial resistance to complement-mediated killing and on the mechanism of action of bactericidal antibody. Curr. Top. Microbiol. Immunol. vol. 121, pp 135-158.

Jones, D.M., Sutcliffe, E.M. and Curry, A. (1991a). Recovery of viable but non-culturable *Campylobacter jejuni*. Journal of General Microbiology, vol. 137, pp 2477-2482.

Jones, K. and Telford, D. (1991b). On the trial of a seasonal microbe. New Scientist, 6 April, pp 34-37.

Kaneko, T., Sato, S., Kotani, H. and 21 authors (1996). Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* spp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. Nucleic Acids Research, vol. 3, pp 109-136.

Kapperud, G. and Rosef, O. (1983). Avian wildlife reservoir of *Campylobacter fetus* subsp. *jejuni*, *Yersinia* spp., and *Salmonella* spp. in Norway. Applied and Environmental Microbiology, vol. 45, no. 2, pp 375-380.

Karlyshev, A.V., Henderson, J., Ketley, J.M. and Wren, B.W. (1998). An improved physical and genetic map of *Campylobacter jejuni* NCTC 11168 (UA580). Microbiology, vol. 144, pp 503-508.

Kedzierska, B. (1978). N-Acetylneuraminic acid: a constituent of the lipopolisaccharide of *Salmonella toucra*. Europian Journal of Biochemistry, vol. 91, pp 545-552.

Keenleyside, W.J., Bronner, D., Jann, K., Jann, B. and Whitfield, C. (1993). Coexpression of colanic acid and serotype specific capsular polysaccharides in *Escherichia coli* strains with group II K antigens. Journal of Bacteriology, vol. 175, pp 6725-6730.

Ketley, J. M. (1995). Virulence of *Campylobacter* species: a molecular genetic approach. Journal of Medical Microbiology, vol. 42, pp 312-327.

Ketley, J.M. (1997). Pathogenesis of enteric infection by *Campylobacter*. Microbiology, vol 143, pp 5 - 21.

Kim, N. W., Bingham, H., Khawaja, R., Louie, H., Hani, E., Neote, K. and Chan, V. L. (1992). Physical map of Campylobacter jejuni TGH9011 and localisation of 10 genetic markers by use of Pulsed-Field Gel Electrophoresis. Journal of Bacteriology, vol. 174, no. 11, pp 3494-3498.

Kim, N.V., Lombardi, R., Bingham, H., Hani, E., Louie, H., Ng, D. and Chan, V.L. (1993). Fine mapping of the three rRNA operons on the updated genomic map of *Campylobacter jejuni* TGH9011 (ATCC 43431). Journal of Bacteriology, vol. 175, pp 7468-7470.

Kimura, A. and Hansen, E.J. (1986). Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolisaccharide and their relationship to virulence. Infection and Immunology, vol. 51, pp 69-79.

Klena, J.D. and Schnaitman, C.A. (1994). Genes for TDP-rhamnose synthesis affect the pattern of lipopolisaccharide heterogenity in *Escherichia coli* K12. Journal of Bacteriology, vol. 176, pp 4003-4010.

Klena, J.D. (1998). Cloning, sequencing, and characterization of the lipopolysaccharide biosynthetic enzymeheptosyltransferaseIgene(waaC) from *Campylobacter jejuni* and *Campylobacter coli*. Gene, vol. 19 , pp 177-185.

Konkel, M.E., Lobert, Y. and Cieplak, W. (1992). Examination of multiple isolates of *Campylobacter jejuni* for evidence of cholera toxin-like activity. In: Nachamkin, I., Blaser, M. J., Tompkins, L. S. (Editors). *Campylobacter jejuni:* current status and future trends. American Society for Microbiology, Washington DC, pp 193-198.

Konkel, M.E. and Klena, J. D. (1995). An investigation into *Campylobacter jejuni*. Grant proposal

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson Jr, D.E. and Cieplak Jr, W. (1997). Identificationand molecular cloning of a gene encoding a fibronectin-binding protein (*CadF*) from *Campylobacter jejuni*. Molecular Microbiology, vol. 24, pp 953-963.

Konkel, M.E., Gray, S.A., Garvis, S.G. and Yoon, J. (1999a). Identification of the enteropatogens *Campylobacter jejuni* and *Campylobacter coli* based on the *cadF* virulence gene and its product. Journal of Clinical Microbiology, vol. 37, no. 3, pp 510-517.

Konkel, M,E., Bond, J.K., Vanessa Rivera-Amill and Steven, G.G. (1999b). Bacterial secreted proteins are required for the internalisation of *Campylobacter jejuni* into cultured mammalian cells. Molecular Microbiology, vol. 32, no. 4, pp 691-701.

Krauss, J.H., Reuter, G., Schauer, R., Weckesser, J. and Mayer, H. (1988). Sialic acid-containing lipopolisaccharides in purple nonsulfur bacteria. Arch. Microbiol.vol. 150, pp 584-589.

Lacey, R.W. (1993). Food-borne bacterial infections. Parasitology, vol. 107, pp 575-593.

Iadron de Guevara, C., Gonzalez, J. and Pena, P. (1994). Bacteraemia caused by *Campylobacter* spp. Journal of Clinical Pathology, vol. 47, pp 174-175.

Levinson, G. and Gutman, G.A. (1987). Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Molecular Biology and Evolution, vol. 4, pp 203-221.

MacLachlan, P.R., Kadam, S.K. and Sanderson, K.E. (1991). Cloning, characterisation, and DNA sequence of the *rfaLK* region for lipopolusaccharide synthesis in *Salmonella typhimurium* LT2. Journal of Bacteriology, vol. 173, pp 7151-7163.

Mandrell, R.E., Griffiss, J.M., Smith, H. and Cole, J.A. (1993). Distribution of a lipooligosaccharide-specific silyltransferase in pathogenic and non-pathogenic *Neisseria*. Microbiol. Pathog. vol. 14, pp 315-327.

Mandrell, R.E., Leese, A.J., Sugai, J.V., Shero, M., Griffiss, J.M., Cole, J.A., Parsons, N.J., Smith, H., Morse, S.A. and Apicella, M.A. (1990). *In vitro* and *in vivo* modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. Journal of Experimental Medicine, vol. 171, pp 1649-1664.

McSweegan, E. and Walker, R.I. (1986). Identification and characterisation of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infection and Immunity, vol. 53, pp 141-148.

Miller, J.F., Dower, W.J. and Tompkins, L.S. (1988). High voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proceedings of the National Academy of Sciences USA, vol. 85, pp 856-860.

Mills, S. D., Aspinall, G. O., McDonald, A. G., Raju, T. S., Kurjanczyk, L. A., and Penner, J. L.(1992). Lipopolysaccharide antigens of *Campylobacter jejuni,* In: Nachamkin, I., Blaser, M. J., Tompkins, L. S. (Editors). *Campylobacter jejuni:* current status and future trends. American Society for Microbiology, Washington DC. pp 223-229.

Mishu, B. and Blaser, M.J. (1994). *Campylobacter jejuni,* Human Immunodeficiency Virus, and the Guillain-Barre Syndrome. Journal of Infectious Diseases, vol. 169, May, pp 1177.

Moran, A.P. (1995). Biological and serological characterisation of *Campylobacter jejuni* lipopolisaccharides with deviating core and lipid A structures. FEMS Immunology and Medical Microbiology, vol. 12, no. 8, pp 288-289.

Moran, A.P., Zahringer, U., Seydel, U., Scholz, D., Stutz, P. and Rietschel, E.T. (1991b). Structural analysis of the lipid A component of *Campylobacter jejuni* CCUG 10936 (serotype O:2) lipopolysaccharide. European Journal of Biochemistry, vol. 198, pp 459-469.

Moran, A-P., Rietschel, E.T., Kosunen, T.U. and Zahringer, U. (1991). Chemical Caracterisation of *Campylobacter jejuni* Lipopolysaccharides Containing N-Acetylneuraminic Acid and 2,3 diamino-2,3-dideoxy-D-glucose. Journal of Bacteriology, vol. 173, no. 2, pp 618-626.

Moran, E.E., Brandt, B.L. and Zollinger, W.D. (1994). Expression of the L8 lipopolisaccharide determinant increases the sensitivity of *Neisseria meningitidis* to serum bactericidal activity. Infection and Immunology, vol. 62, pp 5290-5295.

Moxon, E.R. and Maskell, D. (1992). *Haemophilus influenzae* lipopolisaccharide: the biochemistry and biology of a virulence faktor. In Molecular Biology of Bacterial Infection, Current Status and Future Perspectives. Society for General Microbiology Symposium 49. Hormeache, C.E., Penn, C.W. and Smythe, C.J. (Editors). Cambridge: Cambridge University Press.

Nachamkin, I. (1992). *Campylobacter* and *Arcobacter.* In: Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolken, R.H. (Editors). Manual of Clinical Microbiology, 6th edition. American Society for Microbiology, Washington DC, pp 483-491.

Nachamkin, I., Allos, B.M. and Ho, T. (1998). *Campylobacter* Species and Guillain-Barré Syndrome. Clinical Microbiology Reviews, vol. 11, no. 3, pp 555-567.

Nachamkin, I.K., Bohachik, and Patton, C.M. (1993). Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. Journal of Clinical Microbiology, vol. 34, pp 277-281.

Nikaido, H. and Nakae, T. (1979). The outer membrane of of Gram-negative bacteria. Advances in Microbial Physiology, vol. 20, pp 163-250.

Nikaido, H. and Vaara, M. (1985). Molecular Basis of Bacterial Outer Membrane Permeability. Microbiological Reviews, vol. 49, no. 1, pp 1-32.

Nuijten, P.J.M., Bartels, C., Bleumink-Pluym, N.M.C., Gaastra, W. and van der Zeijst, B.A.M. (1990). Size and physical map of the *Campylobacter jejuni* chromosome. Nucleic Acids Research, vol. 18, no. 21, pp 6211-6214.

Pacha, R.E., Clark, G.W., Williams, E.A. and Carter, A.M. (1988). Migratory birds of central Washington as reservoirs of *Campylobacter jejuni.* Canadian Journal of Microbiology, vol 34, pp 80-82.

Parker, C.T., Kloser, A.W., Schnaitman, C.A., Stein, M.A., Gottesman, G. and Gibson, B.W. (1992). Role of *rfa G* and *rfa P* genes in determining the lipopolysaccharide structure and cell surface properties of *Escherichia coli* K12. Journal of Bacteriology, vol. 174, pp 2525-2538.

Pei, Z. and Blaser, M.J. (1993). PEB1, the Major Cell-binding factor of *Campylobacter jejuni,* is a homolog of the binding component in Gram-negative nutrient transport systems. The Journal of Biological Chemistry, vol. 286, no. 25, September, pp 18717-18725.

Pennie, R.A., Pearson, R.D., Barret, L.J., Lior, H. and Guerrant, R.L. (1986). Susceptibility of *Campylobacter jejuni* to strain specific bactericidal activity in the sera of infected patients. Infection and Immunology, vol. 52, pp 702-706.

Pospeich, A. and Neumann, B. (1995). A versatile Quick-Prep of genomic DNA from Gram-positive bacteria. Trends in Genetics, vol. 11, pp 217-218.

Prendergast, M.M., Lastovica, A.J. and Moran, A.P. (1998). Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain-Barré syndrome exibit mimicry of GM_1 ganglioside. Infection and Immunity, vol. 66, no. 8, pp 3649-3755.

Preston, A., Mandrell, R.E., Gibson, B.W. and Apicella, M.A. (1996). The Lipooligosaccharides of pathogenic Gram-negative bacteria. Critical Reviews in Microbiology, vol. 22, no. 3, pp 139-180.

Preston, M.A. and Penner, J.L. (1989). Characterisation of cross-reacting serotypes of *Campylobacter jejuni*. Canadian Journal of Microbiology, vol. 35, pp 265-273.

Raetz, C.R.H. (1990). Biochemistry of Endotoxins. Annual Reviews of Biochemistry, vol. 59, pp 129-170.

Raetz, C.R.H. (1993). Bacterial Endotoxins: Extraordinary Lipids That Activate Eucaryotic Signal Transduction. Journal of Bacteriology, vol. 175, pp 5745-5753.

Rees, J. H., Gregson, N. A., Griffiths, P. L. and Hughes, R. A. C. (1993). *Campylobacter jejuni* and Guillain-Barre syndrome. Quarterly Journal of Medicine, vol. 86, pp 623-634.

Rees, J.H., Vaughan, R.W., Kondeatis, E. and Hughes, R.A.C. (1995). HLA-class II alleles in Guillain-Barre syndrome and Miller-Fisher syndrome and their association with preceding *Campylobacter jejuni* infection. Journal of Neuroimmunology, vol. 62, pp 53-57.

Reeves, P. (1994). Biosynthesis and assembly of lipopolysaccharde: Bacterial Cell Wall. (Editors. Ghuysen, J.M., Hakenbeck, R.) New Comprehensive Biochemistry. Elsevier Science B.V. vol. 27, chpt. 13, pp 281-317. Elsevier Science B.V. Amsterdam, Netherlands.

Reeves, P., Hobbs, M., Valvano, M.A., Skurnik, M., Whitfield C., Coplin, D., Kido,N., Klena, J., Maskell, D., Raetz, C. and Rick, P.D. (1996). Bacterial lipopolysaccharide synthesis and gene nomenclature. Trends in Microbiology, vol. 4, no.12, December, pp 495-503.

Reina, J., Ros, M.J. and Serra, A. (1994). Susceptibilities to 10 antimicrobial agents of 1,220 *Campylobacter* strains isolated from 1987 to 1993 from faeces of paediatric patients. Antimicrobial Agents and Chemotherapy, vol. 38, pp 2917-2920.

Rest, R.F. and Mandrell, R.E. (1995). Neisserial sialyltransferases and their role in pathogenesis. Microb. Pathog. vol. 19, pp 379-390.

Richardson, P.T. and Park, S.F. (1995). Enterochelin acquisition in *Campylobacter coli*: characterisation of components of a binding-protein-dependant transport system. Microbiology, vol. 141, pp 3181-3191.

Rietschel, E.T. and Brade, H. (1992). Bacterial Endotoxins. Scientific American, vol. 267, pp 54-61.

Roncero, C. and Casadaban, M.J. (1992). Genetic analysis of the genes involved in synthesis of the lipopolisaccharide core in *Escherichia colli* K12: Three operons in the *rfa* locus. Journal of Bacteriology, vol. 174, pp 3250-3260.

Ropper, A.H. (1992). The Guillain-Barre Syndrome. New England Journal of Medicine, vol. 326, no. 17, pp 1130-1136.

Rosef, O., Gondrosen, B., Kapperud, G. and Underdal, B. (1983). Isolation and Characterization of *Campylobacter jejuni* and *Campylobacter coli* from Domestic and Wild Mammals in Norway. Applied and Environmental Microbiology, vol. 46, no. 4, pp 855-859.

Ruiz-Palacios, G.M. (1992). Pathogeneis of *Campylobacter* infection: In vitro models. In I. Nachamkin, Blasier, M.J. and Tompkins, L.S. (Editors), *Campylobacter jejuni*: Current status and future trends. American Society for Microbiology, Washington DC, pp. 158-159.

Salloway, S., Mermel, L.A., Seamans, M., Aspinall, G.O., Nam Shin, J.E., Kurianczyk L.A., and Penner, J.L., (1996). Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD_3. Infection and Immunity, vol. 64, no. 8, pp 2945-2949.

Sambrook, J., Fritch, E.F. and Maniatis, T., (Editors). (1989). Molecular cloning: A Laboratory Manual, 2nd Edition. 3 volumes. Cold Spring Harbor Laboratory Press, Cold Sprin Harbor, New York.

Sanger, F., Hicklen, S. and Couslen, A.R. (1977) DNA sequencing with chain terminating inhibitors. Proceedings of the National Academy of Sciences USA, vol. 74, pp 5463-5467.

Sanger centre. (1998). www.sanger.ac.uk/Project

Schnaitiman, C.A. and Klena, J.D. (1993). Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiological Reviews, vol. 57, pp 655-682.

Schnaitman, C.A., Parker, C.T., Klena, J.D., Pradel, E.L., Paerson, N.B., Sanderson, K.E. and MacLachlan, R. (1991). Physical maps of the *rfa* loci of *Escherichia colli* K12 and *Salmonella typhimurium*. Journal of Bacteriology, vol.173, no. 23, pp 7410-7411.

Schwan, E.T., Robertson, B.D., Brade, H. and Van Putten, J.P.M. (1995). Gonococcal *rfaF* mutants that express Rd_2 chemotype LPS and do not enter epithehal host cells. Molecular Microbiology, vol. 15, pp 267-275.

Siebert, P.D. (1993). Quantitive RT-PCR. Methods and Applications; Book 3, Clonetech Laboratories, Inc.

Sirisena, D.M., Brozek, K.A., MacLachlan, P.R., Sanderson, K.E. and Raetz, C.R.H. (1992). The *rfaC* gene of *Salmonella typhimurium*. Cloning, sequencing and enzymatic function in heptose transfer to lipopolysaccharide. The Journal of Biological Chemistry, vol. 267, pp 18874-18884.

Sirisena, D.M., MacLachlan, P.R., Liu, S.L., Hessel, *A.* and Sanderson, K.E. (1994). Molecular analysis of the *rfaD* gene, for heptose synthesis, and the *rfaF* gene for heptose transfer, in lipopolysaccharide synthesis in *Salmonella typhimunum*. Journal of Bacteriology, vol. 176, pp 2379-2385.

Smith, C.L., Econome, J.G., Schutt, A., Klco, S. and Cantor, C.R. (1987). A physical map of the *Escherichia coli* K12 genome. Science, vol. 236, pp 1448-1453.

Stanier, R.Y., Adelberg, E.A. and Ingraham, J.L. (1984). General microbiology, 4th Edition. Macmillan Press Ltd., London.

Stehr-Green, J.K., Nicholls, C., McEwan, S., Payne, A. and Mitchell, P. (1991). Waterborne outbreak of *Campylobacter jejuni* in Christchurch: the importance of a combined epidemiologic and microbiologic investigation. New Zealand Medical Journal, vol. 104, pp 356-358.

Stonnet, V. and Guesdon, J.L. (1993). *Campylobacter jejuni*: Specific oligonucleotides and DNA probes for use in polimerase chain reaction-based diagnosis. FEMS Immunology and Medical Microbiology, vol. 7, pp 337-344.

Suzuki, S., Kawaguchi, M., Mizuno, K., Takama, K. and Yuki, N. (1994). Immunological properties and ganglioside recognitions by *Campylobacter jejuni*-enterotoxin and cholera toxin. FEMS Immunology and Medical Microbiology, vol. 8, pp 207-212.

Takayama, K., Qureshi, N., Hyver, K., Honovich, J., Cotter, R.J., Mascagni, P. and Schneider, H. (1986). Characterisation of a structural series of lipid A obtained from the lipopolisaccharides of *Neisseria gonorrhoeae.* The Journal of Biological Chemistry, vol. 261, no. 23, pp 10624-10631.

Taylor, D.E. (1992). Genetics *of Campylobacter* and *Helicobacter.* Annual Reviews in Microbioliology, vol. 46, pp 35-64.

Taylor, P.W. (1995). Resistance of Bacteria to Complement: Roth, J.A., Bolin, C.A., Brogden, K.A., Minion, F.C. and Wannemuehler, M.J. (Editors). Virulence Mechanisms of Bacterial Pathogens, 2nd ed.

Turley, E.A. and Roth, S. (1980). Interactions between the carbohydrate chains of hyaluronate and chondroitin sulphate. Nature, vol. 283, pp 268-271.

Upritchard, H.G. (1997). Identification and classification of the *gmhA* gene in *Campylobacter jejuni.* MSc Thesis. University of Canterbury, New Zealand.

van Putten, J.P.M. (1993). Phase variation of lipopolisaccharide directs interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae.* Europian Journal of Biochemistry, vol. 12, pp 4043-4051.

Varloot, L.L., Henrissat, B., Gaboriaud, C., Bissery, V., Morgat, A. and Mornon, J.P. (1990). Hydrophobic cluster analysis: procedures to derive structural and functional information from 2-D-representation of protein sequences. Biochimie, vol. 72, pp 555-574.

Veron, M., and R. Chatelain. (1973). Taxonomic study of the genus *Campylobacter* Sebald and Veron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Veron. Int. J. Syst. Bacteriol. vol. 23, pp 122-134.

van Vliet, A.H.M., Wooldridge, K.G. and Ketley, J.M. (1998). Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. Journal of Bacteriology, vol. 180, no. 20, pp 5291-5298.

Vogel, U., Weinberger, A., Frank, R., Muller, A., Kohl, J., Atkinson, J.P. and Frosch, M. (1997). Complement factor deposition and serum resistance in isogenic capsule and lipoologosaccharide sialic acid mutants of serogroup B *Neisseria meningitidis.* Infection and Immunology, vol. 65, pp 4022-4029.

Walan, A., Dahlgren, C., Kihlstorm, E., Stendahl, O and Lock, R. (1992). Phagocyte killing of *Campylobacter jejuni* in relation to oxidative activation. Acta Pathol Microbiol Immunol Scand. vol. 100, pp 424-430.

Walker, R. I., Caldwell, M.B., Lee, E.C., Guerry, P., Trust, T.J. and Ruiz-Palacios, G.M. (1986). Pathophysiology of *Campylobacter* enteritis. Microbiological Reviews, vol. 50, no. 1, pp 81-94.

Walker, R.I., Rollins, D.M. and Burr, D.H. (1992). Studies of *Campylobacter jejuni* infection in the adult rabbit. In *Campylobacter jejuni*: Current Status and Future Trends, pp. 139- 147.

Walker, R.I., Schmauder-Chock, E.A., Parker, J.L. and Burr, D. (1988). Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. Canadian Journal of Microbiology, vol. 34, pp 1142-1147.

Wallis, M.R. (1994). The pathogenesis of *Campylobacter jejuni.* British Journal of Biomedical Science, vol. 51, pp 57-64.

Wang, Y. and Taylor, D. (1990). Natural Transformation in *Campylobacter* Species. Journal of Bacteriology, vol. 172, no. 2, pp 949-955.

Wassenaar, T.M., Fry, B.N. and van der Zeijst, B.A.M. (1993). Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation. Gene, vol. 132, pp 131-135.

Weckesser, J. and Mayer, H. (1988). Different lipid A types in lipopolisaccharides of phototrophic and related non-phototrophic bacteria. FEMS Microbilogical Reviews, vol. 54, pp 143-154.

Weiser, J.N., Love, J.M. and Moxon, E.R. (1989). The Molecular Mechanism of Phase Variation of *H. influenzae* Lipopolisaccharide. Cell, vol. 59, pp 657-665.

Whitfield, C. (1995). Biosynthesis of lipopolisaccharide O antigens. Trends in Microbiology, vol. 3, pp 178-185.

Wilson, I.G. and Moore, J.E. (1996). Presence of *Salmonella* spp. and *Campylobacter* spp. in shellfish. Epidemiological Infections, vol. 116, pp 147-153.

Wood, A.C., Neil, J.O., Cliona, A.O. and Ketley, J.M. (1999). Cloning, mutation and distribution of a putative lipopolisaccharide biosynthesis locus in *Campylobacter jejuni*. Microbiology, vol. 145, pp 379-388.

Wooldridge, K.G., Williams, P.H. and Ketley, J.M. (1994). Iron responsive genetic-regulation in *Campylobacter jejuni*; cloning and characterisation of a *fur* homolog. Journal of Bacteriology, vol. 176, pp 5852-5856.

Yao, R., Burr, D.H. and Guerry, P. (1997). CheY-mediated modulation of *Campylobacter jejuni* virulence. Molecular Microbiology, vol. 23, pp 1021-1031.

Yao, R.J., Burr, D.H., Doig, P., Trust, T.J., Niu, H.Y. and Guerry, P. (1994). Isolation of motile and nonmotile insertional mutants of *Campylobacter jejuni* – the role of motility in adherence and invasion of eukaryotic cells. Molecular Microbiology, vol. 14, pp 883-893.

Yethon, J.A., Heinrichs,D.E., Monteiro, M.A., Perry, M.B. and Whitfield, C. (1998). Involvment of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolisaccharide and their role in the formation of a stable outer membrane. The Journal of Biological Chemistry, vol. 273, no. 41, pp 26310-26316.

Yuki, N., Taki, T., Takahashi, M., Saito, K., Tai, T., Miyatake, T. and Handa, S. (1994). Penner's serotype 4 of *Campylobacter jejuni* has a lipopolysaccharide that bears a GM1 ganglioside epitope as well as one that bears a GDla epitope. Infection and Immunity, vol. 62, no. 5, pp 2101-2103.

Zamze, S.E., Ferguson, M.A.J., Moxon, E.R., Dwek, R.A. and Rademacher, T.W. (1987). Identification of phosphorylated 3-deoxy-manno-octulosonic acid as a component of *Haemophilus influenzae* lipopolisaccharide. Biochemical Journal, vol. 245, pp 583-587.

APPENDIX I

MEDIA

I.i. GENERAL MEDIA

Unless otherwise stated, all media were sterilised by autoclaving for 20 minutes, 121°C at 120 kPa. Solutions that were labile at this temperature were filter-sterilised through a 0.22 μm filter prior to addition to sterile media.

Luria Bertani Medium (LB)	Per litre
1.0% w/v Bacto tryptone	10g
0.5% w/v yeast extract	5g
0.5% w/v NaCl	5g

Dissolved in dH_2O, pH adjusted to 7.4 with 2M NaOH before autoclaving.

LB Agar (LBA)	Per litre
LB media with addition of 1.5% w/v agar	15g

Nutrient Broth No. 2	Per litre
Beef extract	10g
Peptone	10g
NaCl	5g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving.

Nutrient Broth No. 2 Agar	Per litre
Nutrient Broth No. 2 with addition of 1.5% w/v agar	15g

Mueller-Hinton Agar	Per litre
30% w/v beef infusion	300g
1.75% w/v acid hydrolysate of casein	17.5g
0.15% starch	1.5g
1.7% Bacto-agar	17g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving. For culturing of *Campylobacter*, defibrinated sheep blood (5%) was added after autoclaving.

Brain Heart Infusion Broth	Per litre
Beef heart infusion	25g
Calf brain infusion	20g
Protease peptone	10g
NaCl	5g
Na_2HPO_4:$12H_2O$	2.5g
Glucose	2g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving. For storage of *Campylobacter*, 20% glycerol was added to the medium after autoclaving.

I.ii. SPECIALISED MEDIA

***Campylobacter* Blood-Free Selective Agar Base (Modified CCDA-Preston)**

	Per litre
Oxoid *Campylobacter* Blood-Free Selective Agar Base (Modified CCDA-Preston)	12.5 g

Dissolved in dH_2O and pH adjusted to 7.4 before autoclaving. After sterilisation, 1 ml of cefoperasone solution (3.2 mg/l) was added to the medium.

MacConkey Medium (Mac)	Per litre
Difco MacConkey agar in dH_2O	50g

APPENDIX II

BUFFERS AND SOLUTIONS

II.i. COMMON BUFFERS

Solutions requiring sterilisation were either autoclaved for 20 minutes at 121°C, 120 kPa or filter-sterilised through a 0.22 μm filter. All solutions were stored at ambient temperature unless otherwise stated.

TE	Per litre
10 mM Tris-HCl	1.2 g
1 mM EDTA	0.38 g

Dissolved in dH_2O, pH adjusted to 8.0.

50 × TAE	Per litre
2.5 M Tris base	242 g
0.11% v/v glacial acetic acid	57.1 ml
50 mM Na_2EDTA (pH 8.0)	46.5 g

Dissolved in dH_2O to final volume of 1 litre, pH adjusted to 8.0.

1× TAE

20 ml of 50 × TAE dissolved in dH_2O to a final volume of 1 litre.

10 × TBE	Per litre
0.5 M Tris base	108 g

0.5 M Boric acid	55 g
10 mM Na_2EDTA	9.3 g

Dissolved in dH_2O to final volume of 1 litre, pH adjusted to 8.0.

6 × DNA Loading Buffer for Agarose Gel Electrophoresis

50% v/v Glycerol

0.25% w/v Bromophenol blue

0.25% w/v Xylene cyanol FF

Dissolved in dH_2O.

II.ii. SPECIFIC BUFFERS AND SOLUTIONS

II.ii.a. ALKALINE EXTRACTION OF PLASMID DNA

Solution 1 (Stored at 4°C)

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

Dissolved in dH_2O, autoclaved to sterilise.

Solution II

1% w/v SDS

0.2 N NaOH[1]

Dissolved in dH_2O, not sterilised.

[1] Freshly diluted from 10 N stock

Solution III (Stored at 4°C)

3 M potassium acetate

11.5% v/v glacial acetic acid

Dissolved in dH_2O, resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

II.ii.b. ISOLATION OF GENOMIC DNA

SET buffer

75 mM NaCl

25 mM EDTA (pH 7.5)

20 mM Tris

II.ii.c. SOUTHERN HYBRIDISATION SOLUTIONS

Depurination solution	Per litre
0.2 M HCl	17.2 ml

Prepared using concentrated HCl diluted with ddH_2O.

Denaturation Solution	Per litre
0.5 M NaOH	20 g
1.5 M NaCl	87.7 g

Dissolved in ddH_2O.

Neutralisation Solution	Per litre
0.5 M Tris-HCl (pH 7.0)	76.9 g
3 M NaCl	175.5 g

Dissolved in ddH2O, pH adjusted to 7.0.

20 × SSC (Standard Saline Citrate)	Per litre
3 M NaCl	175 g
0.3 M Trisodium citrate	88 g

Dissolved in ddH2O, pH adjusted to 7.0 with citric acid.

Transfer Solution

20 × SSC, pH 7.0

DIG Buffers and Solutions

Maleic Acid Buffer

M maleic acid

0.15 M NaCl

pH adjusted to 7.5 with concentrated or solid NaOH

Washing Buffer

Add 0.3% v/v Tween 20 to Maleic acid buffer

Blocking Solution

10% v/v blocking reagent

90% v/v maleic acid buffer

Prepared freshly, heated at 37°C to dissolve.

Post-Hybridisation Low Stringency Wash I

2 × SSC

0.1% SDS

Post-Hybridisation High Stringency Wash II

0.5 × SSC

0.1% SDS

Detection Buffer

M Tris-HCl (pH 9.5)

M NaCl

II.ii.d. SDS-PAGE FOR PROTEINS

To make a 15% acrylamide separating gel and 4% stacking gel the following volumes of solutions were mixed.

Table A1.1. SDS-PAGE protein gel recipies

	15% Separating Gel	4% Stacking Gel
40% Acrylamide/Bis stock	3.75 ml	1 ml
1.5 M Tris-HCl (pH 8.8)	3.75 ml	
0.5 M Tris-HCl (pH 6.8)		1.25 ml
10% SDS	0.1 ml	0.1 ml
TEMED	5 µl	15 µl
10% w/v Ammonium persulphate*	25 µl	50 µl
ddH2O	2.37 ml	7.6 ml
Total Volume	10 ml	10 ml

* Ammonium persulphate was made fresh each time.

10% w/v SDS

sodium dodecyl sulphate (SDS)

10 g

Dissolved in 100 ml ddH2O.

0.1% w/v Bromophenol blue

bromophenol blue	10 mg
ddH2O	10 ml

2 × Sample Buffer

4 × upper buffer	12.5 ml
glycerol	20.0 ml

Dissolved in ddH2O to a final volume of 60 ml.

2 × Loading Buffer

2-β-mercaptoethanol	0.5 ml
0.1% Bromophenol blue	0.25 ml
10% SDS	4.0 ml
2 × Sample buffer	5.3 ml

4 × Running Buffer Stock

Tris-base	60 g
glycine	288 g

Dissolved in ddH2O to a final volume of 5 litre.

1 × Running Buffer

4 × Running buffer	1 litre

ddH_2O	3 litres
10% SDS	40 ml

Protein Stain

isopropanol	125 ml
acetic acid	50 ml
ddH_2O	325 ml
Coomassie brilliant blue (R250)	1.25 g

Destain

methanol	100 ml
acetic acid	140 ml

Added ddH_2O to a final volume of 2 litres.

II.ii.e. SDS-PAGE FOR LPS

To make a 18% acrylamide separating gel and 4.5% stacking gel the following volumes of solutions were mixed.

Table A1.2 SDS-PAGE LPS gel recepies

	18% Separating Gel	4.5% Stacking Gel
40% w/v acrylamide	9 ml	2.25 ml
2% w/v methylene bis-acrylamide	3.6 ml	0.4 ml
Gel buffer (4 M Tris-HCl)	5 ml	3.7 ml
100% v/v glycerol	2.1 ml	
ddH_2O	0.3 ml	13.1 ml
TEMED	4 µl	6 µl
10% Ammonium persulphate	40 µl	60 µl
Total volume	20 ml	20 ml

Gel Buffer	per 100 ml
4 M Tris-HCl	48.46 g
0.4% w/v SDS	0.4 g

Dissolve with gentle heating in ddH_2O, adjust pH to 8.45 with concentrated HCl.

LPS Running Buffer	per litre
0.1 M Tris-HCl	12.14 g
0.1 M Tricine	17.92 g
0.1% w/v SDS	1 g

Dissolve in ddH_2O, adjust pH to 8.25 with concentrated HCl.

II.ii.f. SILVER STAINING SOLUTIONS FOR LPS

Fixing Solution

25% v/v isopropanol

7% v/v glacial acetic acid

68% v/v ddH_2O

Oxidation Solution

2.7% v/v fixing solution

0.7% w/v periodic acid

Prepare solution freshly in ddH_2O.

Silver Solution

28 ml 0.1 N NaOH (dilute 1 N stock)

1.25 ml NH_4OH (29.4% stock)

5 ml 20% $AgNO_3$

115 ml ddH2O

Citric Acid Developer

50mg citric acid

0.5 ml formaldehyde (37% stock)

Dissolve in 1 litre of ddH_2O

Reaction Stop Solution

0.35% v/v acetic acid

Prepare solution freshly in ddH_2O

APPENDIX III

Blast Sequence

A. Blast aligments for *C. jejuni* 928 *gmhA*

```
gb|AAD07903.1| (AE000596) phosphoheptose isomerase (gmhA)
[Helicobacter pylori26695]
           Length = 192

 Score =  219 bits (552), Expect = 1e-56
 Identities = 113/184 (61%), Positives = 142/184 (76%), Frame = +1

Query: 7   NLVEKEWQEHQKIIQES-
EILKGQIAKVGELLCECLKKGGKILICGNGGSAADAQHFAAE 183
           NL++KE+  H++ +++S E L+  + +   LL E L+
GKILICGNGGSA+DAQHFAAE
Sbjct: 4
NLIKKEFLAHKEALEKSLEGLQEALKQSVHLLIETLENQGKILICGNGGSASDAQHFAAE 63

Query: 184
LSGRYKKERKALAGIALTTDTSALSAIGNDYGFEFVFSRQVEALGNENDVLIGISTSGKS 363
           L+GRYK ERK L+ I+L TD SAL+AI NDYG+E VF+RQVEALG +NDVLIGISTSG
S
Sbjct: 64
LTGRYKLERKGLSAISLNTDISALTAIANDYGYEEVFARQVEALGVKNDVLIGISTSGNS 123

Query: 364
PNVLEAFKKAKELNMLCLGFSGKGGGMMNKLCDHNLVVPSDDTARIQEMHILIIHTLCQI 543
            NVL+A++KAK+L M  L  +G+ GG M  L D  L+VPSDDT RIQEMHIL+IH LC
Sbjct: 124
KNVLKAYEKAKDLEMKTLSLAGRDGGKMKPLSDMALIVPSDDTPRIQEMHILMIHILCDC 183

Query: 544 IDEGF 558
           I+  F
Sbjct: 184 IERHF 188

sp|P51001|LPCA_ECOLI PHOSPHOHEPTOSE ISOMERASE >gi|984578|dbj|BAA07584|
(D38582) YafI
           [Escherichia coli] >gi|1079568 (U32590) phosphoheptose
           isomerase [Escherichia coli] >gi|1786416 (AE000131)
           phosphoheptose isomerase [Escherichia coli]
           >gi|4902958|dbj|BAA77892.1| (D83536) Phosphoheptose
           isomerase (EC  5.-.-.-). [Escherichia coli]
           Length = 192

 Score =  150 bits (376), Expect = 6e-36
 Identities = 79/159 (49%), Positives = 104/159 (64%), Frame = +1
```

```
Query: 76
IAKVGELLCECLKKGGKILICGNGGSAADAQHFAAELSGRYKKERKALAGIALTTDTSAL 255
           I +   LL +  K GGK+L CGNGGS  DA HFA EL+GRY++ R     IA++ D S
+
Sbjct: 30  IQRAAVLLADSFKAGGKVLSCGNGGSHCDAMHFAEELTGRYRENRPGYPAIAIS-
DVSHI 88

Query: 256
SAIGNDYGFEFVFSRQVEALGNENDVLIGISTSGKSPNVLEAFKKAKELNMLCLGFSGKG 435
           S +GND+GF  +FSR VEA+G E DVL+GISTSG S NV++A   A+E  M  +  +GK
Sbjct: 89
SCVGNDFGFNDIFSRYVEAVGREGDVLLGISTSGNSANVIKAIAAAREKGMKVITLTGKD 148

Query: 436 GGMMNKLCDHNLVVPSDDTA-RIQEMHILIIHTLCQIIDE 552
           GG M    D  + VP    A RIQE+HI +IH L Q+I++
Sbjct: 149 GGKMAGTADIEIRVPHFGYADRIQEIHIKVIHILIQLIEK 188
```

B. Blast aligment for *C. jejuni*928 *lex2B*

gi|507354 (U05670) Lex2B [Haemophilus influenzae]

Length = 247

Plus Strand HSPs:

Score = 80 (36.6 bits), Expect = 5.5e-23, Sum P(3) = 5.5e-23
Identities = 15/27 (55%), Positives = 20/27 (74%), Frame = +1

```
Query:     7 VFIINLERSLDRKEHMKKQIQKLFEKN 87
             +FIINLE+S DRK +M+ Q + LF  N
Sbjct:     6 IFIINLEKSTDRKAYMQAQFELLFSNN 32
```

Score = 140 (64.1 bits), Expect = 5.5e-23, Sum P(3) = 5.5e-23
Identities = 23/43 (53%), Positives = 33/43 (76%), Frame = +1

```
Query:   199 GRELSDGEKACFASHYKLWQECVKLDEPIIILEDDVEFSDEFL 327
             G  L+ G+  C+ASHY +W++CV+LD PII+LEDD +F + FL
Sbjct:    67 GYPLTLGQLGCYASHYSMWEKCVELDYPIIVLEDDAKFKNNFL 109
```

Score = 85 (38.9 bits), Expect = 5.5e-23, Sum P(3) = 5.5e-23
Identities = 18/39 (46%), Positives = 22/39 (56%), Frame = +1

```
Query:   457 AGTQGYVLQVSAAVKFLKYAKNWIYAVDDYMDMFYKHNV 573
             AG  GY L   AA KFL  +K W   VD  MD F+++ V
Sbjct:   155 AGATGYYLTPQAARKFLTQSKEWYLTVDVTMDRFFENKV 193
```

gi|2078280 (U94833) Lob1 [Haemophilus somnus]

Length = 287

Plus Strand HSPs:

Score = 66 (30.2 bits), Expect = 9.4e-22, Sum P(3) = 9.4e-22
Identities = 12/36 (33%), Positives = 22/36 (61%), Frame = +1

```
Query:     7 VFIINLERSLDRKEHMKKQIQKLFEKNPSLKNKLEF 114
             +F+INLE++ +RK  +  Q   L E++P +    +F
Sbjct:    42 IFVINLEKATERKHFISHQFTALQEQHPDIVINYQF 77
```

Score = 132 (60.5 bits), Expect = 9.4e-22, Sum P(3) = 9.4e-22
Identities = 22/43 (51%), Positives = 31/43 (72%), Frame = +1

```
Query:   199 GRELSDGEKACFASHYKLWQECVKLDEPIIILEDDVEFSDEFL 327
             G E++ G+  C+ASHY LW++CV+L +PII+LEDD       FL
Sbjct:   105 GNEITLGQLGCYASHYLLWEKCVQLQQPIIVLEDDAILQPNFL 147
```

Score = 99 (45.4 bits), Expect = 9.4e-22, Sum P(3) = 9.4e-22
Identities = 21/50 (42%), Positives = 30/50 (60%), Frame = +1

```
Query:   457 AGTQGYVLQVSAAVKFLKYAKNWIYAVDDYMDMFYKHNVLNIVKKPLFLK 606
             + T GY L   AA KFL  ++ WIY VD +MD FY+++V  +   P  +K
Sbjct:   195 SNTTGYYLTPQAAQKFLDSSQEWIYNVDIFMDRFYENHVALLGVNPPCVK 244
```

gi|2313935 (AE000592) lipooligosaccharide 5G8 epitope biosynthesis-associated protein (lex2B) [Helicobacter pylori]

Length = 284

Plus Strand HSPs:

Score = 134 (61.4 bits), Expect = 5.0e-16, Sum P(3) = 5.0e-16
Identities = 22/42 (52%), Positives = 30/42 (71%), Frame = +1

```
Query:   199 GRELSDGEKACFASHYKLWQECVKLDEPIIILEDDVEFSDEF 324
             G+ +  GE  C+ASHY LWQ+C++L+E I ILEDD+   D F
Sbjct:    94 GKRMGFGELGCYASHYSLWQKCIELNEAICILEDDIIIKDRF 135
```

```
 Score = 62 (28.4 bits), Expect = 5.0e-16, Sum P(3) = 5.0e-16
 Identities = 13/19 (68%), Positives = 14/19 (73%), Frame = +1

Query:   460 GTQGYVLQVSAAVKFLKYA 516
             GTQGYVL   AA K LKY+
Sbjct:   182 GTQGYVLAPKAAQKLLKYS 200

 Score = 58 (26.6 bits), Expect = 5.0e-16, Sum P(3) = 5.0e-16
 Identities = 11/24 (45%), Positives = 13/24 (54%), Frame = +1
Query:   514 AKNWIYAVDDYMDMFYKHNVLNIV 585
             AK W+  +D  MD  Y H V N V
Sbjct:   201 AKEWVMPIDCVMDRHYWHGVKNYV 224
```

sp|Q50947|LGTB_NEIGO LACTO-N-NEOTETRAOSE BIOSYNTHESIS GLYCOSYL TRANFERASE LGTB

>gi|595811 (U14554) glycosyl transferase [Neisseria gonorrhoeae]

Length = 279

```
 Score = 35.2 bits (79), Expect = 0.59
 Identities = 35/100 (35%), Positives = 49/100 (49%), Frame = +1

Query: 208 LSDGEKACFASHYKLWQECVKLDEP-IIILEDDV---
EFSDEFLNNGVEYIDELLKSKYE 375
           LS  EKACF SH  LW++ +    P I + EDDV   E +++FL       +
Sbjct: 58
LSGVEKACFMSHAVLWEQALDEGVPYIAVFEDDVLLGEGAEQFLAEDTWLQERFDPDSAF 117

Query: 376 YIRLCYLFDKRLYFLSESG--------YYLSIEKLAGTQGYVLQVSAAVKFL 507
            +RL  +F   L   S SG        + L   +  GT GY++   A   FL
Sbjct: 118 VVRLETMFMHVL--TSPSGVADYGGRAFPLLESEHCGTAGYIISRKAMRFFL 167
```

```
sp|Q51117|LGTE_NEIME LACTO-N-NEOTETRAOSE BIOSYNTHESIS GLYCOSYL
TRANFERASE LGTE
           >gi|973187 (U25839) glycosyl tranferase [Neisseria
           meningitidis] >gi|1586803|prf||2204376C lgtE gene
           [Neisseria meningitidis]
           Length = 276

 Score = 36.7 bits (83), Expect = 0.20
 Identities = 31/101 (30%), Positives = 50/101 (48%),  Frame = +1

Query: 208
LSDGEKACFASHYKLWQECVKLDEPIIILEDDVEFSDEFLNNGVEYIDELLKSKYEYIRL 387
           LS+ EKACF SH  LW++ +    P + + +  + +++FL       +   K
+RL
Sbjct: 58
LSEVEKACFMSHAVLWKQALDEGLPYVAVFELGKDAEKFLAEDTWLEERFDKDSAFIVRL 117

Query: 388 CYLF-------DKRLYFLSESGYYLSIEKLAGTQGYVLQVSAAVKFLK 510
             +F       DK L + + S    L  E    GT GY++    A    FL+
Sbjct: 118 ETMFAKVIVRPDKVLNYENRSFPLLESEHW-GTAGYIISREAMRFFLE 164
```

C. Blast aligment for *waaF'*

```
sp|P37692|RFAF_ECOLI ADP-HEPTOSE--LPS HEPTOSYLTRANSFERASE II
>gi|1073586|pir||S47841
           rfaF protein - Escherichia coli >gi|466758 (U00039) rfaF
           [Escherichia coli] >gi|1790050 (AE000440)
           ADP-heptose--lps heptosyltransferase II;
           lipopolysaccharide core biosynthesis [Escherichia coli]
           Length = 348

 Score = 44.9 bits (104), Expect = 1e-04
 Identities = 24/77 (31%), Positives = 42/77 (54%), Gaps = 1/77 (1%)
```

```
Query: 6
RSFLTNDSGPMHLSAVYKVKTVVIFGPTKFTQTSPWQNQNARWVHLVLACMPCMQKTCPL 65
           ++ +TNDSG MH++A      V ++GP+    T P  ++ AR + L+       +
Sbjct: 263 KAIVTNDSGLMHVAAALNRPLVALYGPSSPDFTPPLSHK-
ARVIRLITGYHKVRKGDAAE 321

Query: 66  KHHKCMKDLKPQRVIEE 82
            +H+ + D+ PQRV+EE
Sbjct: 322 GYHQSLIDITPQRVLEE 338
```

sp|P37421|RFAF_SALTY ADP-HEPTOSE--LPS HEPTOSYLTRANSFERASE II
>gi|459161 (U06472)

ADP-heptose:LPS heptosyltransferase II [Salmonella typhimurium]
Length = 348

```
 Score = 44.5 bits (103), Expect = 2e-04
 Identities = 24/77 (31%), Positives = 42/77 (54%), Gaps = 1/77 (1%)

Query: 6
RSFLTNDSGPMHLSAVYKVKTVVIFGPTKFTQTSPWQNQNARWVHLVLACMPCMQKTCPL 65
           ++ +TNDSG MH++A      V ++GP+    T P  ++ AR + L+       +
Sbjct: 263 KAIVTNDSGLMHVAAALDRPLVALYGPSSPDFTPPLSHK-
ARVIRLITGYHKVRKGDTAQ 321

Query: 66  KHHKCMKDLKPQRVIEE 82
            +H+ + D+ PQRV+EE
Sbjct: 322 GYHQSLIDITPQRVLEE 338
```